Modulation of Hepatocyte Protein Synthesis by Endotoxin-activated Kupffer Cells

II. Mediation by Soluble Transferrable Factors

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We have previously reported a diminution of protein synthesis by isolated Sprague–Dawley rat hepatocytes following coculture with lipopolysaccharide-triggered nonparenchymal liver cells (NPC) containing 30–40% Kupffer cells.[1] It is possible that this cell-mediated modulation of hepatocyte function represents an *in vitro* model for hepatic insufficiency occurring in patients with the multiple system organ failure syndrome. In the present report we have determined that supernatant from lipopolysaccharide-triggered NPC was itself capable of inhibiting hepatocyte protein synthesis in a similar fashion. This effect was directly related to the concentration of the supernatant and to the period of exposure to the supernatant. The ability to inhibit hepatocyte protein synthesis by a NPC supernatant suggests that this cell-mediated event is caused at least in part by a relatively stable soluble factor(s) secreted by LPS triggered NPC. Although reagent H_2O_2 will inhibit protein synthesis when added to hepatocyte culture, LPS-stimulated NPC do not release H_2O_2 and do not show chemiluminescence—an *in vitro* correlate of the respiratory burst. Nonspecific protease inhibitors added to the coculture similarly do not influence the system. Combined with other evidence, the soluble mediators do not seem to be the result of oxidative or proteolytic secretions of the effector cells.

IN SPITE OF a significant morbidity and mortality attendant to hepatic failure associated with sepsis or severe trauma,[2-4] little is understood with respect to its causes. Although hypoxemia and hypoperfusion often accompany or precede hepatic insufficiency in this setting, most investigators feel that these factors alone cannot account for the alterations in organ function in patients with the multiple system organ failure syndrome (MSOF).[5-6] In light of recent work from various scientific disciplines describing mechanisms of cellular injury mediated by effector cells of monocyte–macrophage lineage,[1,7-12] we have investigated the role of Kupffer cell-mediated modulation of hepatocyte function in coculture.

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We have previously demonstrated that isolated hepatocytes synthesize protein in a reliable fashion for periods of culture ranging from 24 to 48 hours.[1] When cocultivated with nonparenchymal liver cells (NPC) consisting of 30–40% Kupffer cells, no alterations in hepatocyte protein synthesis was observed. When *Salmonella typhimurium* lipopolysaccharide (LPS) was added to the cell mixture, however, a significant decrease in calculated hepatocyte protein synthesis was seen.[1] The purpose of the present investigation was to characterize further this phenomenon by determining whether or not the effect of LPS-triggered NPC on hepatocyte function could be induced by transferrable soluble mediators released from triggered NPC.

Materials and Methods

Animals

Male Sprague–Dawley rats 4–6 weeks old and 250–300 g (Charles River Breeding Laboratories, Wilmington, MA) were used throughout this experiment. Animals had free access to water and Purina Lab Chow #500 and were not fasted prior to use.

Hepatocyte Isolation

A modification of Seglen's collangenase perfusion technique[13] was used. Rats were anesthetized with Nembutal® and the portal vein was cannulated *in vivo*.

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Perfusion of the liver was initiated with the calcium-free solution described by Seglen while the hepatic artery, vein, and duct, as well as any other attachments, were severed and the liver was removed. Following a 7-minute perfusion period with the calcium-free solution, the liver was perfused at 37 C with Hanks balanced salt solution (HBSS) containing 0.05% collagenase (Sigma #C-0130 Type I, 200 μ/mg) for 10 minutes. The perfused liver was then combed to release the cells. Hepatocytes were obtained from the pellet following centrifugation at 10 × G. Approximately 2.0–3.0 × 10^8 hepatocytes were obtained per animal with a viability rate of at least 90%.

Hepatocyte Culture

Minimal essential medium (MEM) containing the following supplements was used for all cultures: fetal calf serum 7 ml/100 ml, penicillin 10,000 units and streptomycin 10 mg/100 ml, L-glutamine 30 mg/100 ml, insulin 10 μ/100 ml (Regular Ilentin ®I 100 μ/ml, Eli Lilly Co.), and HEPES Buffer® 1.5 ml/100 ml. Hepatocytes were plated in 1 ml of medium at a cell density of 5.0 × 10^4 cells/ml in 5% CO_2 and air at 37 C. Following 90 minutes of culture, the medium was replaced with identical medium with the exception that 2% FCS was used. Hepatocyte viability exceeded 85% for culture periods ranging from 24 to 72 hours.

Nonparenchymal Cell Preparation

Nonparenchymal cells (NPC) were obtained by the method described by Knook and Sleyster.[14] The liver was perfused at 37 C *via* the cannulated portal vein with Gey's balanced salt solution (GBS) (137 mM NaCl, 5 mM KCl, 1.6 mM $CaCl_2$–$2H_2O$, 0.9 mM $MgCl_2 \cdot 6H_2O$, 0.3 mM $MgSO_4 \cdot 7H_2O$, 1.7 mM $Na_2HPO_4 \cdot 2H_2O$, 0.2 mM KH_2PO_4, 2.7 mM Na HCO_3, 5.5 mM glucose; pH 7.4; 275 mOsm). The rate of perfusion was maintained at 10 ml/min. After 2 minutes of perfusion, pronase 0.2% (Sigma #5147) was added to the perfusate. The liver was perfused with pronase for 1 minute, removed, and diced into small pieces in 10 ml of GBS. The pieces were then incubated in 90 ml of GBS plus 0.2% pronase at 37 C for 1 hour while the pH was maintained at 7.4 by the addition of NaOH. Following filtration through wide-mesh gauze, NPC were washed in HBSS and centrifuged at 250 × G for 5 minutes. This procedure yielded 1.2–1.6 × 10^8 cells per rat with viability in excess of 95%. This cell preparation was comprised of 30–40% Kupffer cells by latex bead uptake.[15] Latex beads (0.8 μm) were washed and suspended in HBSS and added to freshly isolated NPC to achieve a latex bead/NPC ratio of 10/1. After 4 hours of incubation, the percentage of cells containing latex beads was determined by light microscopy. NPC were plated at 1.0 × 10^6 cells/ml alone or in coculture with 5.0 × 10^4 hepatocytes in the medium described above with 2% FCS. Their viability following 24-hour culture remained in excess of 95%. Where indicated, lipopolysaccharide (LPS) (from *S. typhimurium,* Ribi Immunochem Research Inc.) was added to NPC at 20 μg/ml.

Coculture Technique

5.0 × 10^4 hepatocytes were precultured for a 24-hour period. The medium was then removed and replaced with an equal volume of identical medium containing 1.0 × 10^6 NPC. LPS (20 μg/ml) was then added to appropriate groups and the coculture continued for a 24-hour period. The rate of hepatocyte protein synthesis was then measured as described below. The amount of protein synthesized by the cocultured NPC was subtracted from the coculture total to calculate the amount of protein synthesized by the hepatocytes as previously described.[1]

Supernatant Transfer

The supernatants were removed from 5.0 × 10^4 hepatocytes, 1.0 × 10^6 NPC, or cocultures from these two cell types in the presence or absence of LPS following 24 hours of culture. The supernatant was centrifuged to remove cellular particles and stored at −70 C until use. Supernatants were then thawed and added to 5.0 × 10^4 hepatocytes that had been precultured for 24 hours. Hepatocytes were exposed to varying concentrations of supernatant for time intervals ranging from 4 to 48 hours. Control hepatocytes were exposed to the medium described above, which had been incubated at 37 C for 24 hours in the presence or absence of LPS.

Protein Synthesis Assay

Following coculture or exposure to supernatant, the medium was removed and replaced with identical medium without FCS. 7 μCi of L-4,5 ^{3}H leucine, 5.0 Ci/mmol, (New England Nuclear) was then added in a 50-lambda volume of normal saline. Four hours after the label was added, incorporation of ^{3}H leucine into protein was stopped by addition of 2 ml of cold 10% trichloroacetic acid (TCA) to each well. Wells were scraped by a rubber policeman and well contents transferred to 4-ml glass test tubes. Samples were washed three times in cold 10% TCA and centrifuged at 700 × G to remove unincorporated ^{3}H leucine. The protein pellet was then solubilized with 0.5 ml of 0.5 mol/L Protosol® tissue solubilizer to which 4 ml of scintillation cocktail was added. ^{3}H leucine incorporation into the solubilized protein was then measured using a Beckman® LS 2800 Scintillation counter.

Neutrophil-rich Peritoneal Cells (PMN)

Sprague–Dawley rats were injected intraperitoneally with 8 ml of 3% sterile thioglycollate broth. Eighteen hours later, the animals were killed, and 20 ml of HBSS supplemented with 7% FCS, penicillin 10,000 units and streptomycin 10 mg/100 ml, and beef lung heparin 500 μ/100 ml was injected intraperitoneally. Following 1 minute of gentle agitation, the peritoneal cavity was opened and the fluid removed by pipette. The PMN were washed three times in HBSS and centrifuged at 250 × G for 5 minutes. During the washing procedure, RBCs were removed by hypotonic lysis; 1.5–2.0 × 10^8 cells were obtained per rat. Differential cell counts using Wright's stain revealed 80% PMN, 1% macrophages, and 19% lymphocytes. Viability exceeded 95% by trypan blue exclusion. Phorbol myristate acetate (PMA) (Sigma #P8139) was added to PMN at 20 μg/ml as indicated to stimulate the release of H_2O_2.

H_2O_2 Determination

H_2O_2 release by NPC and PMN was measured by the method described by Pick et al.,[16] utilizing phenol red as a substrate. Standard curves were created for each experiment using 30% H_2O_2 (Sigma #H-1009). H_2O_2 release from LPS-triggered NPC was measured during the initial 2-hour culture period and, in addition, during a subsequent 24-hour culture period. H_2O_2 release by PMA-triggered PMN was used as a control for the assay. All assays were performed in the same medium as was used for hepatocyte culture.

Bovine Pancreas Trypsin Inhibitor (BPTI)

BPTI was obtained from Sigma (#T9503) and stored at −5 C before use. Various concentrations of BPTI were added to the mixed Kupffer cell and hepatocyte cultures in 50-lambda volumes.

Chemiluminescence Assay

2.0 × 10^6 freshly isolated NPC or PMN were suspended in 2.5 ml of Hanks balanced salt solution in glass counting vials (Kimble Glass). Vials were dark-adapted for 24 hours prior to use. LPS was added (20 μg/ml) to appropriate groups for various periods of time and chemiluminescence was measured in a liquid scintillation counter (Beckman LSC-330) at ambient temperature in a noncoincidence mode. Zymosan A (Sigma Chemical Co.) was opsonized by mixing 3 ml of human serum with 1 ml of a 50-mg/ml zymosan suspension in phosphate-buffered solution. This mixture was incubated for 30 minutes at 37 C, washed twice, and resuspended at a concentration of 12.5 mg/ml. 100 μl were added to 2.0 × 10^6 PMN to provide a positive control for the chemiluminescence assay.

TABLE 1. *Effect of Hepatocyte and Nonparenchymal Cell (NPC) Supernatant on Hepatocyte Protein Synthesis*

Group	Source of Supernatant Added	^{3}H Leucine Incorporation into Protein (cpm ± SD)
1	Medium; no cells	17,532 ± 640
2	Medium + LPS 1μg/ml; no cells	18,129 ± 781
3	Medium + LPS 20 μg/ml; no cells	17,493 ± 942
4	Medium + LPS 100 μg/ml; no cells	19,845 ± 847
5	24 hours hepatocyte culture	20,128 ± 476
6	24 hours NPC culture	15,522 ± 1830*
7	24 hours NPC culture (+ LPS added late)	15,863 ± 1159*
8	24 hours hepatocyte + NPC coculture	16,591 ± 2081
9	24 hours hepatocyte + LPS culture	19,803 ± 237
10	24 hours NPC + LPS culture	10,491 ± 1451†
11	24 hours hepatocyte + NPC + LPS coculture	13,826 ± 897‡
	Direct Hepatocyte + NPC Coculture	
12	Hepatocytes alone	17,532 ± 640
13	Hepatocytes + LPS-triggered NPC	9,871 ± 1328

* $p < 0.05$, compared with hepatocytes not exposed to supernatant (group 1).
† $p < 0.001$, compared with hepatocytes not exposed to supernatant (group 1).
‡ $p < 0.01$, compared with hepatocytes not exposed to supernatant (group 1).

Statistical Analysis

Three wells of cultured cells constituted a group in each experiment. Means from at least three experiments were compared. Variance was expressed as standard deviation. The paired Student's *t*-test was used throughout for statistical analysis.

Results

Effect of LPS on Hepatocyte Protein Synthesis

Since LPS was used to stimulate NPC in this investigation, it was necessary to define the effect of LPS on hepatocyte protein synthesis. As depicted in Table 1 (groups 2–4), 24-hour exposure of hepatocytes to LPS at concentrations ranging from 1.0 μg/ml to 100 μg/ml had no effect on total hepatocyte protein synthesis.

Effect of Nonparenchymal Cell Supernatant on Hepatocyte Protein Synthesis

The effect of supernatant from various cell preparations on isolated hepatocyte protein synthesis is shown in Table 1. Supernatants from acellular media with or without LPS (groups 1–4) did not affect hepatocyte protein synthesis. No effects were detected upon addition

TABLE 2. *Inhibition of Hepatocyte Protein Synthesis by LPS-triggered NPC Supernatant (NPC): Dose Response*

Dilution of NPC + LPS Supernatant	^{3}H Leucine Incorporation into Protein (cpm ± SD)
No supernatant	18,074 ± 731
Undiluted	8,072 ± 415*
1:1 (50%)	9,119 ± 672*
1:3 (25%)	9,097 ± 1043*
1:9 (10%)	11,346 ± 554†
1:99 (1%)	16,241 ± 851

* $p < 0.001$, compared with LPS-treated hepatocytes not exposed to supernatant.
† $p < 0.01$, compared with LPS-treated hepatocytes not exposed to supernatant.

of supernatants from a 24-hour culture of hepatocytes with or without LPS (groups 5 and 9). A significant reduction in hepatocyte protein synthesis followed exposure of hepatocytes to supernatant from LPS-triggered NPC (group 10).

This degree of inhibition of protein synthesis was equal to that resulting from direct coculture of equal numbers of LPS-triggered NPC with hepatocytes (group 13), which we have previously reported.[1] Exposure of hepatocytes to supernatant from NPC that were not triggered with LPS (group 6) resulted in some degree of inhibition of hepatocyte protein synthesis, but this inhibition was significantly less than that seen when LPS-triggered NPC supernatant was used (group 10). In addition, the addition of LPS to the 24-hour-old NPC supernatant did not recreate the effect observed when NPC were exposed to LPS for a 24-hour period (group 7). In no experiment was a decrease in hepatocyte viability seen, as determined by trypan blue exclusion.

Dose Response of LPS-triggered NPC Supernatant Inhibition of Hepatocyte Protein Synthesis

Hepatocytes were exposed to increasing concentrations of LPS-triggerd NPC supernatant for a 24-hour period (Table 2). No diminution of hepatocyte protein synthesis occurred following exposure to 1% supernatant. However, significant inhibition did occur with exposure to 10% supernatant, and this degree of inhibition generally increased as the concentration of supernatant was increased.

Kinetics of Inhibition of Hepatocyte Protein Synthesis after Exposure to LPS-triggered NPC Supernatants

Hepatocytes were exposed to supernatant from 1.0×10^6 NPC that had been cultured in the presence or absence of LPS for 24 hours. The effect of various periods of exposure to these supernatants on hepatocyte protein synthesis is shown in Table 3. Significant inhibition of protein synthesis occurred when hepatocytes were exposed to LPS-triggered supernatant for 18 hours, and the degree of inhibition increased following 24 hours of exposure. No greater inhibition was noted following 48 hours compared with 24 hours of exposure. The difference following 4 hours of exposure was not significant. Some inhibition occurred following exposure to supernatant from untriggered NPC, but to a significantly lesser extent than exposure to LPS-triggered supernatant.

H_2O_2 Release from LPS-triggered NPC

To determine the possible role of the Kupffer cell respiratory burst on hepatocyte malfunction, we measured H_2O_2 release and chemiluminescence by LPS-stimulated NPC. No measureable H_2O_2 was released during the initial 2-hour incubation or during a 24-hour incubation period (Table 4), and no chemiluminescent response was seen (Fig. 1). PMA-triggered polymorphonuclear cells were used as a positive control for the H_2O_2 assay, and zymosan-activated polymorphonuclear cells were used as a control for chemiluminescence.

Effect of Antiproteases on LPS-triggered NPC-mediated Inhibition of Hepatocyte Protein Synthesis

We have examined the role of one other possible soluble mediator in this cell-mediated event: evidence

TABLE 3. *Inhibition of Hepatocyte Protein Synthesis by LPS-triggered NPC Supernatant: Time Course*

Source of Supernatant Added	Leucine Incorporation into Protein (Per cent of Hepatocyte + LPS Control) ± SD			
	4 Hours*	18 Hours	24 Hours	48 Hours
None (hepatocytes alone)	100 ± 12	100 ± 12	100 ± 16	100 ± 7
None (hepatocytes + LPS)	100 ± 5	100 ± 4	100 ± 12	100 ± 10
24 hours NPC culture	91 ± 15	100 ± 10	83 ± 19	81 ± 4†
24 hours NPC + LPS culture	96 ± 12	74 ± 8‡	62 ± 17§	61 ± 12†

* Period of exposure to added supernatant.
† $p < 0.05$, compared with LPS treated hepatocytes not exposed to supernatant.
‡ $p < 0.01$, compared with LPS treated hepatocytes not exposed to supernatant.
§ $p < 0.001$, compared with LPS treated hepatocytes not exposed to supernatant.

TABLE 4. *H_2O_2 Release by LPS-triggered Nonparenchymal Cells (NPC)*

	Nanomoles H_2O_2 Produced/1.0 × 10^6 Cells (±SD)			
	NPC	NPC + LPS (20 μg/ml)	PMN	PMN + PMA (20 μg/ml)
H_2O_2 released during initial 2 hours of culture	0.0	0.0	4.0 ± 0.5	57.5 ± 3.8
Cumulative H_2O_2 released during 24 hours of culture	0.0	0.0		

for neutral proteases playing only a limited role in this coculture system is depicted in Table 5. Various concentrations of BPTI, known to inhibit most proteases, were added to hepatocytes alone, LPS-triggered NPC, and cocultures of both cells. Exposure to BPTI did not significantly alter the NPC-mediated diminution of hepatocyte protein synthesis even at high concentrations.

Discussion

Hepatic insufficiency associated with sepsis or severe trauma may occur alone or as a component of the multiple system organ failure (MSOF) syndrome.[2-6] The pathogenesis of liver failure in this clinical setting is poorly understood, but many factors probably play a role. We have recently postulated that hepatocyte malfunction may be mediated by cell–cell interaction between the cells of monocyte–macrophage lineage lining the hepatic sinusoids (Kupffer cells) and the adjacent hepatocytes. The possibility that such an event could take place was confirmed by our observations that a decrease in protein synthesis by isolated rat hepatocytes followed coculture with unstimulated peritoneal macrophages[17] and LPS-triggered Kupffer cells.[1] *In vivo* injury of hepatocytes has also been reported in animal models in which macrophage or Kupffer cell-activating factors have been injected. For example, hepatocyte regeneration is seen after Kupffer cell ingestion of silica particles *in vivo*.[18] In addition, an increase in mortality following endotoxemia occurred in rats when Kupffer cells were stimulated by *in vivo* glucan administration.[19] Alterations in hepatic metabolism of various substances have been reported as a consequence of stimulation of intrahepatic macrophages.[20] Ferluga and Allison reported that chronic *Corynebacterium parvum* injection of rats resulted in diffuse granuloma formation within the liver that was associated with marked increases in mortality following endotoxin administration.[21] Vermillion et al.[22] and Miller et al.[23] both demonstrated an increase in intrahepatic mononuclear cells in patients with jaundice and sepsis, supporting the concept of a macrophage/Kupffer cell-mediated modulation of hepatocytes.

In vitro models of macrophage-[7-12,17] and Kupffer cell-[1,24] mediated modulation of target cells permit investigation of the possible mechanisms involved. Although the majority of cell-mediated cytotoxicity assays have utilized the release of a surface radiolabel by tumor cell lines as a measure of cytolysis,[7-12,24] recent studies have documented more subtle nonlethal alterations in enzyme function in nonneoplastic cells during cell–cell interaction.[25,26] In our investigations we have used the rate of protein synthesis as an assay for hepatocyte functional modulation, in part because of its increased sensitivity compared with cytolysis and also because of its applicability to the clinical state of hepatic insufficiency, which is characterized by alterations in protein metabolism. We do not mean to imply, by the use of this assay, that decreased protein synthesis necessarily indicates irreversible hepatocyte damage.

The results presented in this report suggest that the factor(s) responsible for the LPS-triggered NPC-mediated diminution of hepatocyte protein synthesis in coculture are stable soluble mediators that are capable of recreating the effect following transfer of the supernatant. This observation provides crucial information regarding the nature of the factor(s) involved. Specifically, the ability to transfer this effect suggests that it is not due primarily to unstable molecules, such as the active oxygen intermediates that have been described as effectors of cell-mediated cytotoxicity in several *in vitro* models.[8,27-29] In addition, we have been unable to document H_2O_2 release from LPS-triggered NPC, and these cells do not exhibit chemiluminescence in response to LPS, an assay for the production of active oxygen intermediates.

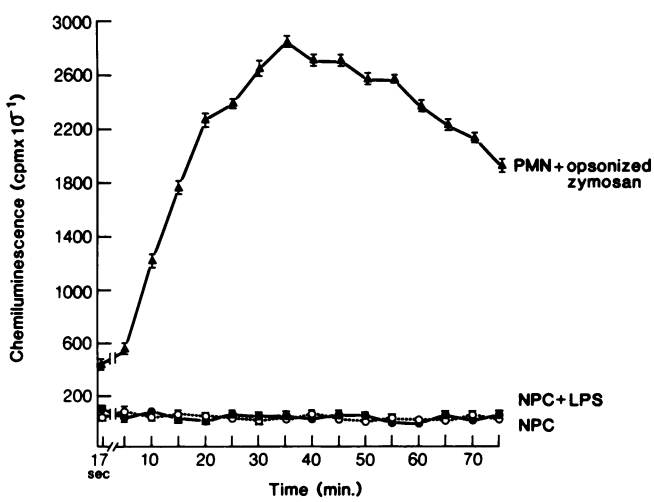

FIG. 1. Effect of lipopolysaccharide triggering on nonparenchymal liver cell chemiluminescence.

TABLE 5. *Effect of Bovine Pancreas Trypsin Inhibitor (BPTI) on LPS-triggered NPC-mediated Inhibition of Hepatocyte Protein Synthesis*

Concentration of BPTI (μg/ml)	Hepatocytes Alone	LPS-stimulated NPC	Hepatocytes + LPS-stimulated NPC in Coculture	Calculated Hepatocytes Alone
0	13,348 ± 594	2,364 ± 94	6,229 ± 686	3,865 ± 686*
1	11,920 ± 893	2,850 ± 187	6,068 ± 530	3,218 ± 530*
10	10,096 ± 126	2,580 ± 176	5,754 ± 259	3,174 ± 259*
100	11,637 ± 2,329	2,685 ± 383	7,573 ± 270	4,888 ± 291*

* $p < 0.001$ compared with hepatocytes alone.

Transferability of this effect suggests that previously described soluble mediators of target cell cytotoxicity or modulation, such as neutral proteases,[30] prostaglandins,[30] or interleukin-1,[31,32] may be responsible for this event. Protease inhibition, however, did not impede the effect. The accompanying paper[33] provides evidence that one of the products of NPC stimulation, namely the monokines (possibly interleukin-1) are responsible—at least in part. We also have preliminary evidence using purified Kupffer cell preparations that it is the Kupffer cell, rather than nonmacrophage components of our NPC preparation, that is responsible for hepatocyte protein modulation (West, unpublished observations).

In our previous report[1] we did not observe a consistent decrease in hepatocyte protein synthesis following coculture with untriggered NPC. In the absence of LPS, however, in this investigation, we did observe a limited inhibition following exposure to untriggered NPC supernatant. The degree of inhibition was significantly less than that caused by exposure to LPS-triggered NPC. These results are consistent with our previous observations that unstimulated peritoneal macrophages can modulate hepatocyte protein synthesis during coculture.[17] It is likely that apparently untriggered NPC-containing Kupffer cells do in fact release some mediators, perhaps in response to the techniques of *in vitro* preparation (*i.e.*, exposure to pronase, fetal calf serum, or plastic plates) and that the limited inhibition of hepatocyte protein synthesis is a result of exposure to lower concentrations of secretory products. We know that the difference between the untriggered NPC supernatant and the LPS-triggered supernatant is not due to the continued presence of LPS during the period of hepatocyte exposure, because no augmentation of inhibition by untriggered NPC supernatant was observed when LPS was added at the time the hepatocytes were exposed to the supernatant (Table 1).

These results, in context with numerous investigation by others in various fields, support the concept that modulation of hepatocyte function and even hepatocyte injury might result from stimulation of the autochthonous reticuloendothelial system. In contrast to the usual notion that Kupffer cells simply protect hepatocytes from potentially noxious substances in the sinusoidal circulation, it is likely that Kupffer cells release mediators in response to these stimuli, which can act in turn to alter the function of adjacent hepatocytes. This phenomenon may account in part for the alteration of hepatocellular function seen in hepatic insufficiency in patients with the multiple organ failure syndrome. Our data do not and cannot prove this hypothesis, but if we could not demonstrate the effect, the hypothesis would have to be discarded.

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