Pronase destroys the lipopolysaccharide receptor CD14 on Kupffer cells

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Ikejima, Kenichi, Nobuyuki Enomoto, Vitor Seabra, Ayako Ikejima, David A. Brenner, and Ronald G. Thurman. Pronase destroys the lipopolysaccharide receptor CD14 on Kupffer cells. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G591–G598, 1999.—CD14 is a lipopolysaccharide (LPS) receptor distributed largely in macrophages, monocytes, and neutrophils; however, the role of CD14 in activation of Kupffer cells by LPS remains controversial. The purpose of this study was to determine if different methods used to isolate Kupffer cells affect CD14. Kupffer cells were isolated by collagenase (0.025%) or collagenase-Pronase (0.02%) perfusion and differential centrifugation using Percoll gradients and cultured for 24 h before experiments. CD14 mRNA was detected by RT-PCR from Kupffer cell total RNA as well as from peritoneal macrophages. Western blotting showed that Kupffer cells prepared with collagenase possess CD14; however, it was absent in cells obtained by collagenase-Pronase perfusion. Intracellular calcium in Kupffer cells prepared with collagenase was increased transiently to levels around 300 nM by addition of LPS with 5% rat serum, which contains LPS binding protein. This increase in intracellular calcium was totally serum dependent. Moreover, LPS-induced increases in intracellular calcium in Kupffer cells were blunted significantly (40% of controls) when cells were treated with phosphatidylinositol-specific phospholipase C. Therefore, it was absent in cells obtained by collagenase-Pronase perfusion. Moreover, treatment with Pronase rapidly cleaves CD14 from Kupffer cells, whereas collagenase perfusion 1 h after addition of LPS, an effect potentiated over twofold by serum; however, serum did not increase TNF-α mRNA in cells isolated via collagenase-Pronase perfusion. Moreover, treatment with Pronase decreased CD14 on mouse macrophages (RAW 264.7 cells) and Kupffer cells. These findings indicate that Pronase cleaves CD14 from Kupffer cells, whereas collagenase perfusion does not, providing an explanation for why Kupffer cells do not exhibit a CD14-mediated pathway when prepared with procedures using Pronase. It is concluded that Kupffer cells indeed contain a functional CD14 LPS receptor when prepared gently.

GUT-DERIVED ENDOTOXIN plays a pivotal role in the development of alcohol-induced liver injury using the chronic enteral ethanol feeding model in the rat (Tsukamoto-French). For example, Adachi et al. (2) demonstrated that elimination of endotoxin by gut sterilization with enteral antibiotics prevented early alcohol-induced liver injury. Moreover, Nanji et al. (18) demonstrated that suppression of bacterial overgrowth using lactobacillus also prevented liver injury in a similar model by decreasing endotoxin levels in portal blood. Furthermore, activation of Kupffer cells by gut-derived endotoxin is an essential step for the development of early alcohol-induced liver injury. Activation of Kupffer cells leads to the production of cytokotoxic mediators such as tumor necrosis factor-α (TNF-α), interleukin-1 and -6, eicosanoids, oxygen radicals, and nitric oxide. Indeed, inactivation of Kupffer cells by GdCl3 treatment (1) or with an antibody for TNF-α (12) prevented early alcohol-induced liver injury in the Tsukamoto-French model. However, the precise receptors by which Kupffer cells are activated by endotoxin remain unclear.

Lipopolysaccharide (LPS), a component of the outer wall of gram-negative bacteria, is a major endotoxin (19), and macrophages and neutrophils contain several different LPS receptors on their cell surface. CD14 is a functional LPS receptor that regulates cytokine production in macrophages (26). It is a phosphatidylinositol-anchored protein that is easily cleaved from cells by phosphatidylinositol-specific phospholipase C (PI-PLC). Membrane bound CD14 does not recognize LPS alone at low concentrations but rather binds LPS and LPS binding protein (LBP), which is present in serum, forming a LBP-LPS-mCD14 ternary complex (6, 26). Because of the requirement for LBP, CD14-mediated pathways are serum dependent; however, it is not clear whether CD14 plays a role in cytokine and eicosanoid production by Kupffer cells. Several recent reports support the hypothesis that CD14 is important for the development of liver injury caused by acute (4) and chronic alcohol (23) and cholestasis (24). On the other hand, activation of NF-κB (3) and TNF-α production (16) by LPS in isolated Kupffer cells has been reported to be serum independent. Together, these studies lead to the idea that the response to LPS in isolated Kupffer cells is not dependent on CD14. Therefore, the purpose of this study was to test the hypothesis that methodological considerations might explain this apparent paradox. Specifically, it was demonstrated here that preparation of Kupffer cells with Pronase cleaved CD14...
from the Kupffer cell membrane. Preliminary accounts of this work have appeared elsewhere (13).

**MATERIALS AND METHODS**

Isolation and culture of rat Kupffer cells. Female Sprague-Dawley rats weighing between 200 and 250 g were used for all experiments. All animals were given humane care in compliance with National Institutes of Health and Institutional Guidelines. Here, Kupffer cells were isolated from the rat liver by collagenase perfusion and differential centrifugation using Percoll (Pharmacia, Upsala, Sweden) as described elsewhere with slight modifications, the standard procedure in this laboratory. Briefly, the liver was perfused in situ through the portal vein with Ca2+- and Mg2+-free Hanks’ balanced salt solution (HBSS) containing 0.5 mM EGTA at 37°C for 5 min at a flow rate of 26 ml/min. Subsequently, perfusion was with HBSS containing 0.025% collagenase IV (Sigma Chemical, St. Louis, MO) at 37°C for 5 min (collagenase perfusion). Because the groups who report CD14 independence used Prone (3, 16), 0.02% Prone (protease, Sigma Chemical) was added to the perfusate (collagenase-Prone perfusion) to make preparations for comparison. After digestion, the liver was excised and cut into small pieces in the collagenase buffer previously described. The suspension was filtered through nylon gauze, and the filtrate was centrifuged twice at 50 g at 4°C for 3 min to remove parenchymal cells. The nonparenchymal cell fraction was washed with buffer and centrifuged on a density cushion of Percoll at 1,000 g for 10 min to obtain the Kupffer cell fraction, followed by washing with buffer again. The viability of isolated Kupffer cells by trypan blue exclusion was >90%. Cells were plated in plastic culture dishes (Corning, Corning, NY) or onto 25-mm glass coverslips at a concentration of 5 × 10^5 cells/coverslip and cultured in RPMI 1640 medium (GIBCO Laboratories Life Technologies, Grand Island, NY) supplemented with 25 mM HEPES, 10% fetal bovine serum (FBS), and antibiotics (100 U/ml of penicillin G and 100 µg/ml of streptomycin sulfate). To increase purity, nonadherent cells were removed by exchanging culture medium 1 h after plating. More than 95% of adherent cells phagocytosed latex beads, indicating that they were Kupffer cells. Cells were cultured at 37°C in 5% CO2 for 24 h before experiments unless otherwise noted.

Preparation of rat peritoneal macrophages. Rats were given an intraperitoneal injection of casein hydrolysate (5% wt/vol, 75 ml/kg) 3 days before cell preparation, and peritoneal cells were collected by lavaging the abdominal cavity with 30 ml HBSS. The cell suspension was passed through sterile gauze and centrifuged at 500 g for 7 min at 4°C to obtain a cell pellet. Cells were washed twice with HBSS and cultured in plastic culture dishes in RPMI 1640 medium containing 10% FBS, 25 mM HEPES, and antibiotics. Nonadherent cells were removed by exchanging the medium 1 h after plating.

Culture of RAW 264.7 cells. RAW 264.7 cells, a mouse macrophage cell line, were obtained from American Type Culture Collection (Rockville, MD) and were cultured in DMEM (GIBCO Laboratories Life Technologies) containing 10% FBS and antibiotics at 37°C in 5% CO2. Medium was exchanged with fresh medium without FBS before experiments.

RNA preparation, RT-PCR, and Northern blotting. Total RNA from cultured cells was prepared using TRIzol reagent (Life Technologies, Grand Island, NY). Concentration and integrity of RNA were determined by measuring absorbance at 260 nm and electrophoresis on 1% agarose gels.

For RT-PCR, 1 µg of total RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (GIBCO Laboratories Life Technologies) and an oligo(dT), primer. The cDNA (1 µl) obtained was amplified using Taq DNA polymerase, and specific forward and reverse primers were used as follows: for CD14 forward (5′-GTG CTC CTG CCC AGT GAA GAG AT-3′) and reverse (5′-GAT CTG TCT GAC AAC CCT GAG T-3′), yielding a 267-bp product size (5); for Kupffer cell receptor (KCR) forward (5′-ATG AAG GAG GCG GAA CTG AAC-3′) and reverse (5′-TCA GCT CTG GTC CTG TCT GGC-3′), yielding a product size of 1653 bp (11); for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward (5′-TGA AGG TCG GAG ACC AGT GTG-3′) and reverse (5′-CAT GTG GCC CAT GAG TTC GTG-3′), yielding a product size of 983 bp (25). The enzyme and dNTPs were added to the reaction mixture after a 4-min denaturation period; thereafter, 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s followed by final extension at 72°C for 7 min were performed using GeneAmp PCR System 9600 (Perkin Elmer, Norwalk, CT). The size and amount of PCR products were verified by electrophoresis in 1% agarose gels.

The steady-state levels of mRNA for CD14 and TNF-α were detected by Northern blot hybridization. Total RNA (5–10 µg) was electrophoresed in formaldehyde-denaturing 1% agarose gels and transferred onto nylon membranes. Membranes were prehybridized in 50% formamide, 5× saline-sodium citrate (SSC), 1× Denhardt’s solution, and 10 µg/ml salmon sperm DNA at 42°C and hybridized with random prime-labeled probe for CD14 or TNF-α at 42°C overnight. Membranes were then washed with 2× SSC, 0.1% SDS at 50°C for 30 min, and 0.1× SSC, 0.1% SDS at 55°C for 30 min, and autoradiography was performed using X-OMAT films. Subsequently, membranes were stripped by washing with 0.1× SSC and 0.1% SDS at 65°C and reprobed for the housekeeping gene GAPDH.

Western blotting for CD14. Protein extracts were prepared from cultured RAW 264.7 cells or Kupffer cells by homogenizing in a cell lysis buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.02% sodium azide, 1 µg/ml aprotinin, 100 µg/ml phenylmethylsulfonyl fluoride, and 1% Triton X-100. Protein concentration was determined spectrophotometrically using a Bradford assay kit (Bio-Rad, Hercules, CA). Then, 25 µg of protein (RAW 264.7 cells) or 5 µg of protein (Kupffer cells) were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Membranes were blocked by Tris-buffered saline-Tween containing 5% skim milk, incubated with a mouse anti-rat myeloid cell ED-9 antibody (MCA 620; Serotec, Oxford, UK), which recognizes denatured Ig potentially present in Kupffer cell membranes. Subsequently, membranes were blotted with a horseradish peroxidase (HRP)-conjugated secondary antibody (rabbit anti-mouse IgG-HRP or anti-goat IgG-HRP). A chemiluminescence substrate (enhanced chemiluminescence reagent) was used for detection of specific bands. No band was detectable with a Western blot of primary antibodies leading to the conclusion that there is no cross-link between the primary antibody and denatured Ig potentially present in Kupffer cell membranes.

Measurement of intracellular Ca2+. Intracellular Ca2+ in individual Kupffer cells was measured using the fluorescent Ca2+ indicator dye fura 2 and a microspectrofluorometer (Photon Technology International, South Brunswick, NJ) interfaced with an inverted microscope (Diaphot, Nikon, Japan) as reported previously (14). Kupffer cells cultured on coverslips were incubated in modified Hanks’ buffer ([in mM] 115 NaCl, 5 KCl, 0.3 Na2HPO4, 0.4 KH2PO4, 5.6 glucose, 0.8 MgSO4, 1.26 CaCl2, 15 HEPES, pH 7.4) containing 5 µM fura 2-acetoxymethyl ester (Molecular Probes, Eugene, OR), and 0.03% Pluronic F-127 (BASF Wyandotte, Wyandotte, MI) at room temperature for 60 min. Coverslips plated with Kupffer cells were preincubated with 1 µM fura 2-acetoxymethyl ester for 45 min at 37°C. Coverslips were placed on the stage of an inverted microscope, and continuous images (30 frames/sec) were obtained using a medium magnification objective (40×, dry). A 450-nm excitation light was used for fura 2 excitation, and the fluorescence emission was recorded at 503 nm (excitation at 340 nm) and 510 nm (excitation at 380 nm). Signals were averaged over 10 frames using a Macintosh computer and NIH Image software (Wayne Rasband, National Institutes of Health, Bethesda, MD). The signal was normalized to the average fluorescence signal and the ratio of fluorescence was calculated for each cell.
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 (8). Intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) was determined from the equation

\[ [Ca^{2+}]_i = K_d \frac{(R - R_{\text{min}})/(R_{\text{max}} - R)}{(F_0/F_s)} \]

where \( F_0/F_s \) is the ratio of fluorescent intensities evoked by 380-nm light from fura 2 pentapotassium salt loaded in cells using a buffer containing 3 mM EGTA and 1 µM ionomycin ([Ca²⁺]₀) or 10 mM Ca²⁺ and 1 µM ionomycin ([Ca²⁺]ₘ). R is the ratio of fluorescent intensities at excitation wavelengths of 340 and 380 nm, and Rₘ and Rₘ are values of R at [Ca²⁺]ₘ and [Ca²⁺]₀, respectively. The values of these constants were determined at the end of each experiment, and a dissociation constant (\( K_d \)) of 135 nM was used (7).

Statistical analysis. All results are expressed as means ± SE. Statistical differences between means were determined using Student’s t-test. \( P < 0.05 \) was selected before the study to reflect significance.

### RESULTS

Kupffer cells prepared with collagenase but not with collagenase-Pronase contain CD14 protein. To clarify whether Kupffer cells contain CD14 mRNA, cells prepared with collagenase alone were probed for CD14 mRNA by RT-PCR (Fig. 1A). Isolated Kupffer cells as well as peritoneal macrophages contained CD14 mRNA 3 and 24 h after plating. RT-PCR for KCR (II), which is a Kupffer cell-specific glycoprotein receptor of unknown function, showed that total RNA from isolated Kupffer cells contained this specific mRNA. To check the integrity of CD14 mRNA, total Kupffer cell RNA was analyzed by Northern blot hybridization (Fig. 1B). Indeed, isolated Kupffer cells contained mRNA for CD14, indicating that Kupffer cells expressed CD14 mRNA constitutively. Next, to confirm whether Kupffer cells contain CD14 protein, protein extracts from isolated Kupffer cells were analyzed by Western blotting using a mouse anti-rat ED9 antibody, which recognizes rat CD14 (Fig. 1C). Kupffer cells prepared by collagenase perfusion expressed CD14, which is a 55-kDa protein, both 3 and 24 h after incubation (Fig. 1C, lanes 1 and 2).
However, specific bands for CD14 were absent when cells were prepared by collagenase-Pronase perfusion (Fig. 1C, lanes 3 and 4).

LPS-induced increases in intracellular Ca\(^{2+}\) in isolated Kupffer cells. LPS increases intracellular Ca\(^{2+}\) in isolated Kupffer cells in the presence of rat serum, which contains LBP (26). Here, this serum dependence of intracellular Ca\(^{2+}\) was compared in cells prepared using collagenase or collagenase-Pronase (Fig. 2). LPS (10 µg/ml) added in the absence of serum did not increase intracellular Ca\(^{2+}\) in Kupffer cells prepared by collagenase perfusion (Fig. 2A). However, when LPS was added with 5% rat serum, intracellular Ca\(^{2+}\) increased in cells isolated by collagenase perfusion with maximal levels reaching values around 300 nM (Fig. 2B). In sharp contrast, LPS did not increase intracellular Ca\(^{2+}\) in cells prepared by collagenase-Pronase perfusion even in the presence of serum (Fig. 2C). However, ATP (100 µM) increased intracellular Ca\(^{2+}\) to similar levels irrespective of the method of preparation, proving that cells were viable and functional.

Because it is known that PI-PLC cleaves CD14 from the plasma membrane, the effect of PI-PLC on LPS-induced increases in intracellular Ca\(^{2+}\) was evaluated (Fig. 3). Maximal increases in intracellular Ca\(^{2+}\) due to LPS reached values around 230 nM in the presence of rat serum in cells prepared with collagenase. However, LPS-induced increases in intracellular Ca\(^{2+}\) were largely blunted when cells were preincubated with PI-PLC (0.5 U/ml), with maximal levels reaching only about 40% of control values. ATP, however, increased intracellular Ca\(^{2+}\) in PI-PLC-treated cells to levels similar to untreated controls, indicating that PI-PLC did not affect cell viability.

LPS-induced increases in TNF-\(\alpha\) mRNA in isolated Kupffer cells. Serum dependency of LPS-induced increases in TNF-\(\alpha\) mRNA in isolated Kupffer cells was evaluated by Northern blot analysis (Fig. 4). Rat serum per se did not increase TNF-\(\alpha\) mRNA. However, in the presence of LPS (1 ng/ml), rat serum (0.1–1%) increased TNF-\(\alpha\) mRNA in Kupffer cells isolated by collagenase perfusion in a dose-dependent manner. Next, the dose dependency of LPS in the induction of TNF-\(\alpha\) mRNA in Kupffer cells was studied (Fig. 5A). Serum-dependent increases in TNF-\(\alpha\) mRNA were observed clearly when cells prepared with collagenase were treated with low doses of LPS (1 ng/ml; Fig. 5B). However, TNF-\(\alpha\) mRNA was increased even in the absence of serum when higher doses of LPS (10 ng/ml) were used. In contrast, others have shown (16) that TNF-\(\alpha\) production is serum independent when cells...
were prepared with collagenase-Pronase perfusion, a finding confirmed here (Fig. 5C).

Effect of polyinosinic acid on LPS-induced increases in TNF-α mRNA in isolated Kupffer cells. To determine if the serum-independent induction of TNF-α mRNA by LPS involved the scavenger receptor, Kupffer cells were pretreated with polyinosinic acid, a scavenger receptor inhibitor. Figure 6 is a representative Northern blot for TNF-α mRNA 1 h after LPS (10 ng/ml) in Kupffer cells isolated by collagenase perfusion alone; however, it disappeared in the absence of serum was blunted by polyinosinic acid to about one-half the levels of untreated controls.

Effect of Pronase on CD14 expression in RAW 264.7 cells and Kupffer cells. To determine if Pronase can indeed remove CD14 from macrophages, whole cell protein extracts from a mouse macrophage cell line (RAW 264.7) or rat Kupffer cells were analyzed by Western blotting (Fig. 7A). As expected, untreated RAW 264.7 cells expressed CD14; however, CD14 progressively disappeared in a time-dependent manner when cells were incubated with 0.02% Pronase for 0.5–10 min (Fig. 7B, left). Similar results were obtained when Kupffer cells were incubated with 0.02% Pronase for 5–10 min at 37°C (Fig. 7B, right).

DISCUSSION
Role of CD14 in Kupffer cells is paradoxical. Because CD14 has been described as a putative LPS receptor on macrophages, many reports have suggested that it participates in the pathophysiology of endotoxic shock (17) and organ damage (20). However, the role of CD14 in Kupffer cells has been controversial. It has been reported that CD14 protein levels were increased in liver macrophages 4 days after bile duct ligation (24). It has also been shown that acute ethanol increased both CD14 mRNA and protein (4). Furthermore, CD14 and LBP mRNA were increased in livers following chronic intragastric ethanol delivery to rats (23). These observations support the hypothesis that CD14 plays a pivotal role in the development of endotoxin-related liver injury. On the other hand, Bellezzo et al. (3) reported that NF-κB activation by LPS, which is important in the transcriptional upregulation of inflammatory cytokines such as TNF-α, was serum independent in isolated Kupffer cells. Furthermore, it has been reported that TNF-α production by isolated Kupffer cells was serum independent and that endocytosis played a pivotal role in TNF-α synthesis (16). These reports suggested that Kupffer cells in the normal liver lack CD14-mediated pathways. In this study it was demonstrated that differences in cell preparation methods are critical for detection of CD14 (see below).

Pronase degrades CD14 from Kupffer cells. Seglen (21) first described the isolation of liver parenchymal cells using collagenase, which gentles digests liver so that it is easy to obtain fragile hepatocytes. Subsequently, Knook and Sleyster (15) reported isolation of Kupffer and endothelial cells from liver using Pronase E, which destroyed hepatocytes allowing the nonparenchymal cell fraction to be purified. More recently, Pronase has been used by many investigators because the nonparenchymal cell yield is much higher compared with preparations using collagenase alone. Our laboratory, however, has avoided use of Pronase because of the possibility that it affects membrane receptor proteins. Specifically, the voltage-dependent Ca2+ channel in Kupffer cells described Hijioka et al. (10) was shown in preliminary studies to be depolarized only when Kupffer cells were prepared gently with collagenase alone (unpublished observation). This led us to hypothesize that Pronase may affect other membrane receptors such as CD14 on Kupffer cells. To test this hypothesis, two different isolation methods were compared. Indeed, CD14 protein was preserved on Kupffer cells isolated by collagenase perfusion alone; however, it disappeared totally when cells were prepared with collagenase-Pronase (Fig. 1C). Strikingly, low doses of Pronase (0.02%) destroyed almost 90% of CD14 on RAW 264.7 macrophages and Kupffer cells in only 5–10 min (Fig. 7). Serum dependency of induction of TNF-α mRNA due to LPS also disappeared completely when Kupffer cells were prepared with Pronase (Fig. 5C). These findings are consistent with the hypothesis that Pronase degrades CD14 from the cell surface. Obviously, the CD14-mediated pathway is not present when Kupffer cells are prepared using Pronase.

Kupffer cells constitutively express CD14 as a functional LPS receptor. It is also not clear whether Kupffer cells express CD14 under normal conditions. Although CD14 in liver macrophages is increased in pathological conditions (i.e., LPS injection, cholestasis, and alcoholic liver injury), one possible explanation was that recruitment of monocytes-macrophages, which express CD14, is involved in the mechanism by which CD14 is upregulated in the liver. This idea was supported by the fact that CD14 was not detected in untreated controls in a study evaluating cholestasis (24). However, here it was demonstrated that Kupffer cells indeed express CD14 in the absence of pathology (Fig. 1). Kupffer cells expressed mRNA for CD14 without any stimulation.

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Fig. 4. Serum dependency of LPS-induced increases in tumor necrosis factor-α (TNF-α) mRNA in Kupffer cells isolated by collagenase perfusion. Kupffer cells isolated by collagenase perfusion were cultured 24 h before experiments. Cells were incubated with 1 ng/ml LPS in 0–1% rat serum for 1 h, and total RNA was prepared. Total RNA (5 μg) was analyzed by Northern blot hybridization for TNF-α. Subsequently, membranes were reprobed for the housekeeping gene GAPDH. Typical experiment was repeated 3 times.
Furthermore, CD14 protein was detectable by Western blot analysis in protein extracts from Kupffer cells prepared by collagenase perfusion alone (Fig. 1C). Taken together, it is concluded that Kupffer cells express both CD14 mRNA and protein constitutively under normal conditions. Whether the use of Pronase explains the lack of positive results in other laboratories is not clear.

Although CD14 is important in LPS-induced cytokine production in other types of macrophages, it was uncertain whether the CD14-mediated pathway is involved in cytokine production in Kupffer cells. To address this question, serum dependency of induction of TNF-α mRNA by LPS was investigated. Rat serum was used here to determine CD14-mediated pathways because it contains LBP, which forms a complex with LPS that is recognized by CD14 (26). As expected, increases in TNF-α mRNA levels by low doses of LPS were dependent on serum when Kupffer cells were prepared with collagenase alone (Figs. 4 and 5A), supporting the hypothesis that the CD14-mediated pathway is involved in the induction of TNF-α mRNA in

![Figure 5](http://ajpgi.physiology.org/)

Fig. 5. Dose dependency of LPS on TNF-α mRNA in Kupffer cells isolated by collagenase or collagenase-Pronase perfusion. A: experimental design is as in Fig. 3 except cells were incubated with 1–10 ng/ml LPS with and/or without 1% rat serum for 1 h. B: ratio of TNF-α to GAPDH from densitometry of data in A was plotted. ●. Data without serum; ■ data with serum. C: Kupffer cells isolated by collagenase-Pronase perfusion were incubated with LPS (1 ng/ml) with and/or without 1% rat serum for 1 h. Ratio of TNF-α to GAPDH was calculated based on densitometric data. Data represent percent increase due to serum over values with LPS obtained in absence of serum. GAPDH is the housekeeping gene. Representative data were repeated 3 times.
Kupffer cells. Furthermore, increases in intracellular Ca\(^{2+}\) by LPS in Kupffer cells isolated by collagenase perfusion were also serum dependent (Fig. 2). This LPS-induced increase in intracellular Ca\(^{2+}\) most likely occurs via CD14, since PI-PLC, which cleaves CD14 from the cell surface, largely (>50%) prevented this increase (Fig. 3). However, the possibility that receptors other than CD14 are affected cannot be excluded completely. Moreover, Pronase completely prevented the increase in intracellular Ca\(^{2+}\) due to LPS (Fig. 2), supporting the hypothesis that Pronase efficiently removes CD14 from the cell surface.

CD14-independent induction of TNF-\(\alpha\) in Kupffer cells: role for scavenger receptor. Although CD14 plays an important role in TNF-\(\alpha\) production due to LPS, there are several different receptors that recognize LPS. In this study serum-independent induction of TNF-\(\alpha\) mRNA was also observed when the dose of LPS was increased (Fig. 5, A and B). The ratio of TNF-\(\alpha\) mRNA induction in serum vs. no serum decreased from over 2.0 to around 1.4 as LPS was increased from 1 to 10 ng/ml. It has been reported that scavenger receptors on macrophages are involved in the recognition and clearance of LPS (9). Polynosinic acid, a scavenger receptor inhibitor, blunted LPS-induced increases in TNF-\(\alpha\) mRNA to approximately one-half of control levels (Fig. 6), indicating that the scavenger receptor participates in TNF-\(\alpha\) production in Kupffer cells. Bellezzo et al. (3) reported serum- and CD14-independent activation of NF-\(\kappa\)B due to LPS in Kupffer cells prepared using Pronase. Moreover, Lichtman et al. (16) reported that TNF-\(\alpha\) production was serum independent in Kupffer cells prepared using Pronase. These findings are consistent with the idea that LPS induces TNF-\(\alpha\) via scavenger receptors in the absence of CD14. On the other hand, Hampton et al. (9) reported that scavenger receptors are not involved in TNF-\(\alpha\) secretion. Importantly, it is obvious that Kupffer cells prepared using Pronase, which cleaves CD14 from the cell surface, do not demonstrate CD14-mediated production of TNF-\(\alpha\).

In conclusion, Kupffer cells play an important role in the pathophysiology of the liver including alcohol-induced injury. Because endotoxin is one of the most important stimulants of Kupffer cells, CD14 most likely plays a pivotal role in the activation of this cell. It is concluded that the method of isolation is important in studies of the function of this receptor and its signaling pathways.
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