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Lipoprotein-bound LPS induces cytokine tolerance in hepatocytes

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Bacterial endotoxin (LPS) elicits dramatic responses in the host including elevated plasma lipid levels due to the increased synthesis and secretion of triglyceride (TG)-rich lipoproteins by the liver. We postulate that this cytokine-induced hyperlipoproteinemia, clinically termed the 'lipemia of sepsis', represents an innate, non-adaptive host immune response to infection. Data in support of this hypothesis include the capacity of TG-rich lipoproteins (VLDL and chylomicrons, CM) to bind and neutralize LPS. Herein, we present evidence that CM-bound LPS attenuates the hepatocellular response to pro-inflammatory cytokines. Primary rodent hepatocytes pretreated with CM–LPS complexes for 2 h demonstrated a near 70% reduction in cytokine-induced NO production as compared to non-pretreated control cells ($P \geq 0.04$). Whereas hepatocytes were maximally tolerant to cytokine stimulation 6 h after CM–LPS pretreatment, the cells spontaneously regained cytokine responsiveness within 40 h. The induction of cytokine tolerance in hepatocytes follows the internalization of CM–LPS complexes and is a process regulated by the LDL receptor. CM–LPS complexes failed to induce cytokine tolerance in hepatocytes wherein lipoprotein receptor activity was inhibited with high dose receptor associated protein (30 μg/ml). Similarly, CM-bound LPS did not induce tolerance in hepatocytes from *ldlr*$^{-/-}$ mice. Thus, the biochemical or genetic inhibition of LDL receptor activity effectively prevented the CM-mediated induction of the cytokine tolerant phenotype. In conclusion, the lipemia of sepsis likely represents a mechanism by which the host combats sporadic, non-life-threatening episodes of endotoxemia. Also, it may indicate a negative regulatory mechanism for the hepatic response to sepsis, serving to effectively down-regulate the acute phase response. A better understanding of how TG-rich lipoproteins modulate the host response to LPS could yield novel biological insights with important clinical implications, including the development of lipid-based therapies for bacterial infections.

Introduction

Bacterial endotoxin (LPS) elicits dramatic responses in the host including elevated plasma lipid levels due to the increased synthesis and secretion of triglyceride (TG)-rich lipoproteins by the liver. This cytokine-induced hyperlipoproteinemia, clinically termed the 'lipemia of sepsis', was customarily thought to represent the mobilization of lipid stores to fuel the host response to infection.[1,2] However, since lipoproteins can also bind and neutralize LPS, we hypothesized that TG-rich lipoproteins (VLDL and chylomicrons, CM) are also components of an innate, non-adaptive host immune response to infection.

Data in support of our hypothesis include: (i) the ability of TG-rich lipoproteins to bind and neutralize LPS; (ii) the observation that hyperlipoproteinemia is protective against LPS-induced toxicity in various models of sepsis; and (iii) the capacity of CM-bound LPS to attenuate the hepatocellular response to pro-inflammatory cytokines.[3–6] The protective capacity of lipoproteins is associated with the increased clearance of circulating LPS by the liver and the re-direction of the toxic macromolecule from Kupffer cells to hepatocytes.[7,8] While the capacity of TG-rich lipoproteins to bind and neutralize

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LPS can be accurately predicted by the phospholipid content of the lipid particles, additional protein constituents of the lipid particle also play an important role in the process. Apolipoprotein E (ApoE), consistent with its ability to regulate lipoprotein binding and uptake by the liver, has been shown to significantly increase the clearance of LPS by hepatocytes,[7] and to protect against endotoxemia in rats.[9] In addition, ApoE knockout mice are more susceptible to LPS-induced lethality than controls, despite elevated plasma lipid levels.[9,10] Taken together, these observations identify ApoE as a vital component of lipoprotein-mediated protection against LPS.

Recently, our laboratory has focused attention on the capacity of CM-bound LPS to attenuate the hepatocellular response to pro-inflammatory cytokines. Specifically, we have shown that infusion of chylomicron-bound LPS (CM–LPS) into rats induces a state of cytokine tolerance in hepatocytes as measured by decreased NO production.[5] Accordingly, cultured hepatocytes exposed to CM–LPS complexes *in vitro* also demonstrated a markedly reduced response to subsequent stimulation by a mixture of TNF-α, IL-1β and IFN-γ. This noted decrease in cytokine-induced NO production is associated with the decreased activation of NF-κB, a ubiquitous transcription factor critical for the expression of numerous inflammatory genes.[11,12] Moreover, TG-rich lipoproteins have been shown to increase the hepatocellular endocytosis of LPS through a receptor-mediated process. Therefore, we have postulated that the lipemia of sepsis, in addition to its homeostatic function, plays a protective role during sepsis.[13] By mobilizing lipid stores, the increased level of TG-rich lipoproteins not only fuels the increased energy demands of the body, but also simultaneously helps protect the host from the deleterious effects of LPS, in part by inducing cytokine tolerance in hepatocytes.

The kinetics of cytokine tolerance in hepatocytes as well as the receptors involved in the process are not known. TG-rich lipoproteins are internalized by hepatocytes through a process mediated by the LDL receptor (LDLR) and the LDL receptor-related protein (LRP). This led us to postulate that the induction of cytokine tolerance in hepatocytes by CM–LPS complexes is also under the control of these receptors. Both receptors can effectively bind and internalize chylomicrons, but under normal conditions the LDLR does so with greater efficiency and speed than does the LRP.[14] Therefore, the LDLR is primarily responsible for the binding and internalization of chylomicrons, with the LRP likely serving as a secondary or back-up mechanism. For example, in subjects with a genetic defect in LDLR function, the LRP mediates the internalization of chylomicrons into hepatocytes. However, since the LRP has slower internalization kinetics, these subjects experience a prolonged postparandial hyperchylomicronemia. Thus, to test the hypothesis that the induction of cytokine tolerance in hepatocytes depends on functional lipoprotein receptors, we examined the kinetics of cytokine tolerance and the subsequent effect of biochemical and genetic manipulations of LDLR activity on this process.

Materials and Methods

Isolation and preparation of hepatocytes

Hepatocytes were isolated from male Sprague-Dawley rats (225–300 g; Simonson, Gilroy, CA, USA) by Liberase® perfusion (Boehringer-Mannheim, Indianapolis, IN, USA), as previously described, and purified via centrifugal elutriation (Beckman Avanti J-20).[8]

Preparation of synthetic chylomicron–LPS complexes

Synthetic chylomicrons were made and combined with recombinant human $ApoE_3$ as described by Redgrave *et al.*[15,16] To make synthetic chylomicron–LPS complexes (sCM–LPS), the lipid emulsion was incubated with LPS (1 μg/20 mg TG) and 10% (v/v) lipoprotein-free fetal bovine serum at 37°C for 3 h. Emulsions were kept under nitrogen at 4°C and used within 5 days of their preparation. Synthetic remnant particles measured 108 ± 45 nm in diameter as determined by electron microscopy.

Study on the kinetics of the hepatocellular response to cytokines after pretreatment with sCM–LPS

To determine the effect of pretreatment time on cytokine tolerance, hepatocytes were pretreated with sCM–LPS (5 mg TG/ml) at 37°C for 2, 4, or 6 h, washed 3 times with PBS, and incubated with fresh medium for 16 h. Hepatocytes were then stimulated with the combination of TNF-α (500 U/ml), IL-1β (100 U/ml), and INF-γ (100 U/ml). After 24 h, NO production was measured using the Griess reaction.

To examine the effect of the length of the recovery period on cytokine tolerance, hepatocytes were pretreated at 37°C with sCM–LPS for 2 h, washed and stimulated with the cytokine mixture 2, 6, 16, 24, and 40 h after the pretreatment. At 24 h after cytokine stimulation, NO production was measured.

Effect of receptor associated protein (RAP) on the response of hepatocytes to cytokines after pretreatment with sCM–LPS complexes

To determine the relative roles of the LDLR and the LRP, receptor-associated protein (RAP) was used at a

low dose to inhibit the LRP and at a high dose to inhibit both the LDLR and LRP. RAP was added to the cell medium at a concentration of 4 μg/ml to block the LRP, or 30 μg/ml to block both the LRP and the LDLR. After 30 min, sCM–LPS complexes were added and pretreatment was continued for an additional 2 h at 37°C. The hepatocytes were then washed with PBS; fresh medium added and cytokine-induced NO production measured after 16 h.

*Response of LDLR-deficient (*ldlr$^{-/-}$*) hepatocytes to cytokines after pretreatment with sCM–LPS complexes*

The role of the LDLR on the induction of cytokine tolerance was further studied by pretreating *ldlr*$^{-/-}$ hepatocytes with sCM–LPS complexes. Hepatocytes were isolated from B6.129 *ldlr*$^{-/-}$ mice (Jackson Laboratory, Bar Harbor, FL, USA) by Liberase® perfusion through the inferior vena cava, filtered through a single layer of sterile gauze, and further purified by discontinuous density gradient centrifugation with metrizamide (15 and 30%; Sigma, St Louis, MO, USA) at 1000 *g* for 40 min. Hepatocytes were subsequently pretreated with sCM–LPS for 2 h and 8 h at 37°C, or sCM–LPS and low-dose RAP (4 μg/ml) for 8 h. Cells were then washed, further incubated in fresh medium for 16 h, stimulated by the cytokine mixture and NO production measured.

Adenoviral restoration of LDL receptors in ldlr$^{-/-}$ *hepatocytes*

ldlr$^{-/-}$ mouse hepatocytes were infected with purified adenovirus expressing recombinant human LDL receptor (AdCMVhLDL) at 0–50 multiplicities of infection (MOI) for 1 h. At 24 h after infection, the viability of hepatocytes was measured via AlamarBlue™ (AccuMed International, Westlake, OH, USA). LDLR expression was assessed via immunoblot using a mouse anti-LDL primary antibody (Oncogene, San Diego, CA, USA) and a goat anti-mouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The optimal MOI was determined by comparing the level of LDLR expression with the associated toxic effect on the virus-infected hepatocytes. Hepatocytes were infected at an MOI of 10 and pretreated with sCM–LPS complexes for 2 h, washed, incubated with fresh medium for 16 h, and stimulated with the cytokine mixture. After 24 h, NO production was measured.

Statistical analysis

Data are presented as the mean ± SEM. A two-way, unpaired *t*-test was used for statistical evaluation of the continuous parametric results and $P \geq 0.05$ was considered significant.

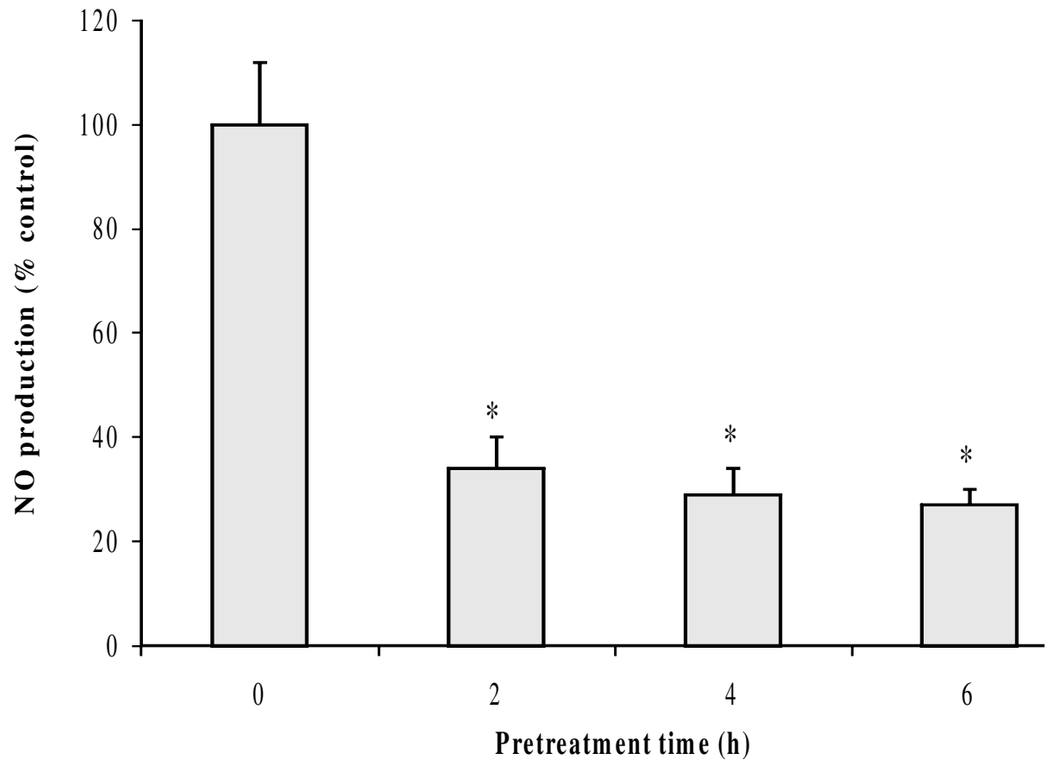

Fig. 1. Pretreatment with sCM–LPS for 2 h is sufficient for maximal attenuation of cytokine-stimulated NO production. Rat hepatocytes were pretreated with sCM–LPS complexes for 0, 2, 4, or 6 h. After 16 h, cells were stimulated with a mixture of pro-inflammatory cytokines (TNF-α, IL-1β, IFN-γ). After a further 24 h, NO production was measured and presented as the percent of the control group (non-pretreated, cytokine stimulated). Data represent the mean ± SEM of 6 samples from two separate experiments in each group, *$P \geq 0.04$ compared to the zero time point.

RESULTS

CM-bound LPS induces cytokine tolerance in hepatocytes

A pretreatment period of 2 h with CM–LPS complexes was sufficient to induce a significant reduction in the hepatocellular response to cytokines (Fig. 1). Increasing the length of the pretreatment time did not result in any further attenuation of the hepatocyte response to pro-inflammatory cytokines. Pretreatment of hepatocytes with either LPS or CM alone had no effect on cytokine responsiveness (data not shown).[17]

Hepatocellular NO production was significantly reduced 2 h after pretreatment with sCM–LPS complexes with the maximal effect observed after approximately 6 h (Fig. 2). After 40 h pretreatment with sCM–LPS complexes, the hepatocellular response to cytokines was indistinguishable from that of untreated control cells.

Inhibition of hepatic lipoprotein receptors prevents induction of cytokine tolerance

Inhibition of the LRP alone with low-dose RAP (4 μg/ml) did not affect the attenuated response to cytokine stimulation (Fig. 3). However, inhibiting both the LRP and LDLR by high-dose RAP (30 μg/ml) prevented the induction of cytokine tolerance following pretreatment with sCM–LPS complexes for 2 h. Pretreatment of hepatocytes from *ldlr*$^{-/-}$

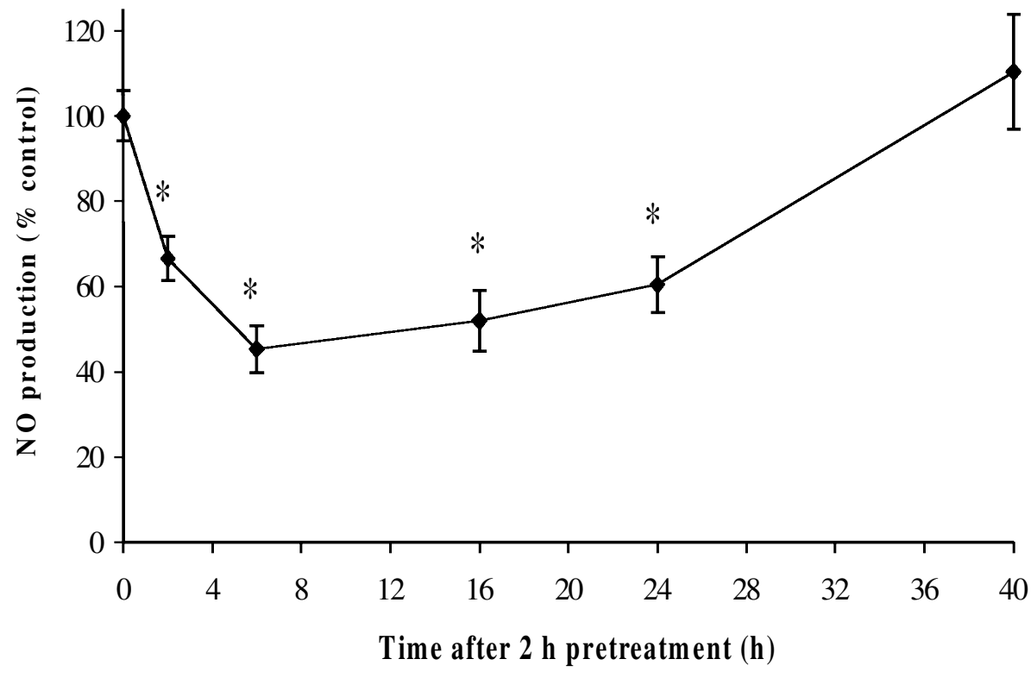

Fig. 2. Attenuation of cytokine-stimulated NO production is maximal 6 h after pretreatment with sCM–LPS. Hepatocytes were pretreated with sCM–LPS complexes for 2 h, and then stimulated at different time points thereafter as shown. NO production was measured and presented as the percent of control (non-incubated, stimulated). Data represent mean ± SEM of 9 samples from three separate experiments, *$P \geq 0.02$ compared to the zero time point.

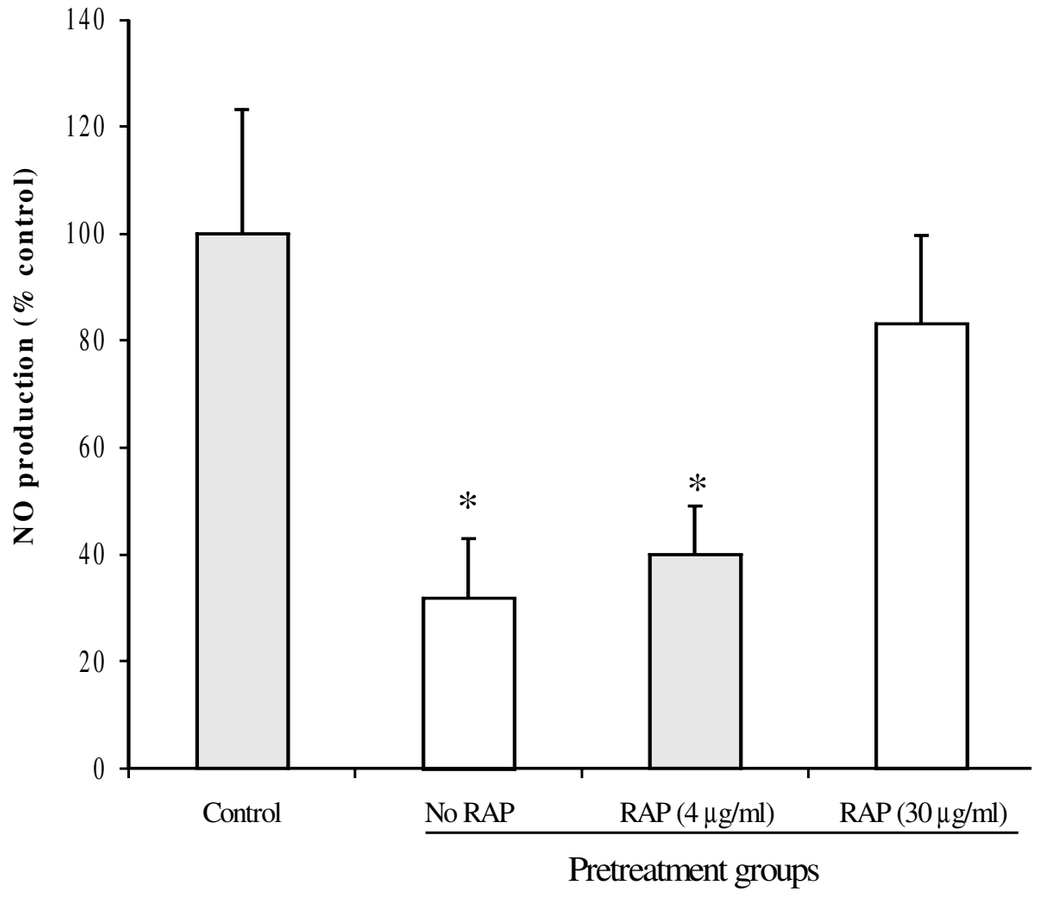

Fig. 3. Addition of RAP blocks the effect of pretreating hepatocytes with sCM–LPS complexes. Primary rat hepatocytes were pretreated with sCM–LPS complexes (5 mg TG/ml) alone or with RAP (4 µg/ml or 30 µg/ml) for 2 h. After 16 h, the cells were stimulated with cytokines and NO production was measured. Each group consists of 6 samples from two separate experiments, *$P \geq 0.001$ compared to control, non-pretreated stimulated cells.

mice with sCM–LPS complexes for 2 h did not induce cytokine tolerance (Fig. 4). However, by increasing the length of the pretreatment period to 8 h, presumably providing sufficient time for sCM–LPS complexes to effectively interact with the LRP receptor, cytokine tolerance was again demonstrated by the pretreated hepatocytes. The addition of low-dose RAP to the pretreatment medium to block the LRP function inhibited induction of cytokine tolerance in hepatocytes, even after the prolonged 8-h pretreatment with sCM–LPS complexes.

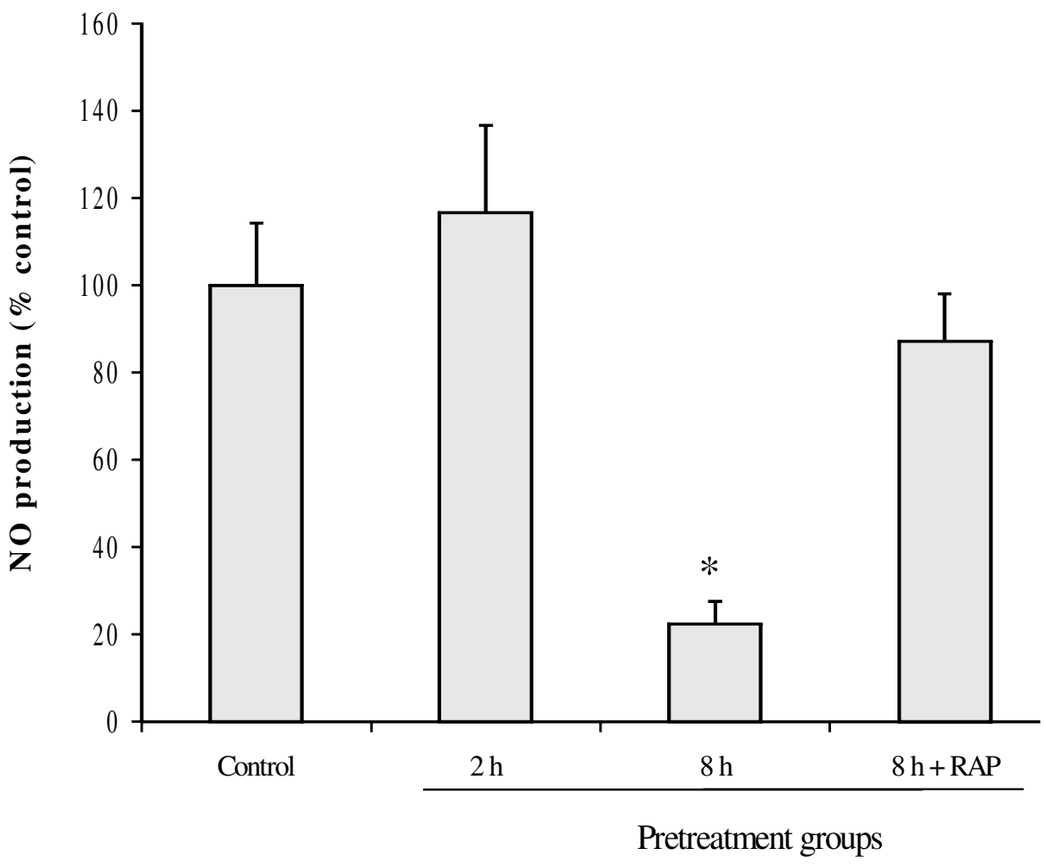

Fig. 4. Hepatocytes lacking the LDLR do not exhibit cytokine tolerance following pretreatment with sCM–LPS complexes. *ldlr*$^{-/-}$ mouse hepatocytes were pretreated with sCM–LPS complexes for 2 h (2 h), 8 h without RAP (8 h), and 8 h with 4 mg/ml of RAP (8 h + RAP). Cells were stimulated with a mixture of cytokines 16 h after pretreatment and NO production was measured. Data represent the mean ± SEM of 9 samples from three separate experiments. *$P = 0.008$ compared to control, non-pretreated stimulated cells.

Effect of restoring the LDLR in ldlr$^{-/-}$ *hepatocytes on cytokine tolerance*

A marked increase in LDLR expression on the membrane of hepatocytes from *ldlr*$^{-/-}$ mice was observed at a MOI of 10 (Fig. 5A). In addition, restoration of the LDLR by adenoviral infection was associated with induction of cytokine tolerance following pretreatment with sCM–LPS complexes for 2 h (Fig. 5B). Thus, adenoviral infection effectively 'reconstituted' LDLR expression on the membrane of hepatocytes from *ldlr*$^{-/-}$ mice which was associated with induction of cytokine tolerance following pretreatment with sCM–LPS complexes.

Discussion

The data demonstrate that CM-mediated cytokine tolerance in hepatocytes is a time- and dose-dependent process that requires functional lipoprotein receptors. The tolerant response to stimulatory cytokines was transient in nature, evident after only 2 h of pretreatment and reached a maximal effect after approximately 6 h. At 40 h after pretreatment, the response of hepatocytes to pro-inflammatory cytokines was similar to that of non-pretreated cells. Either

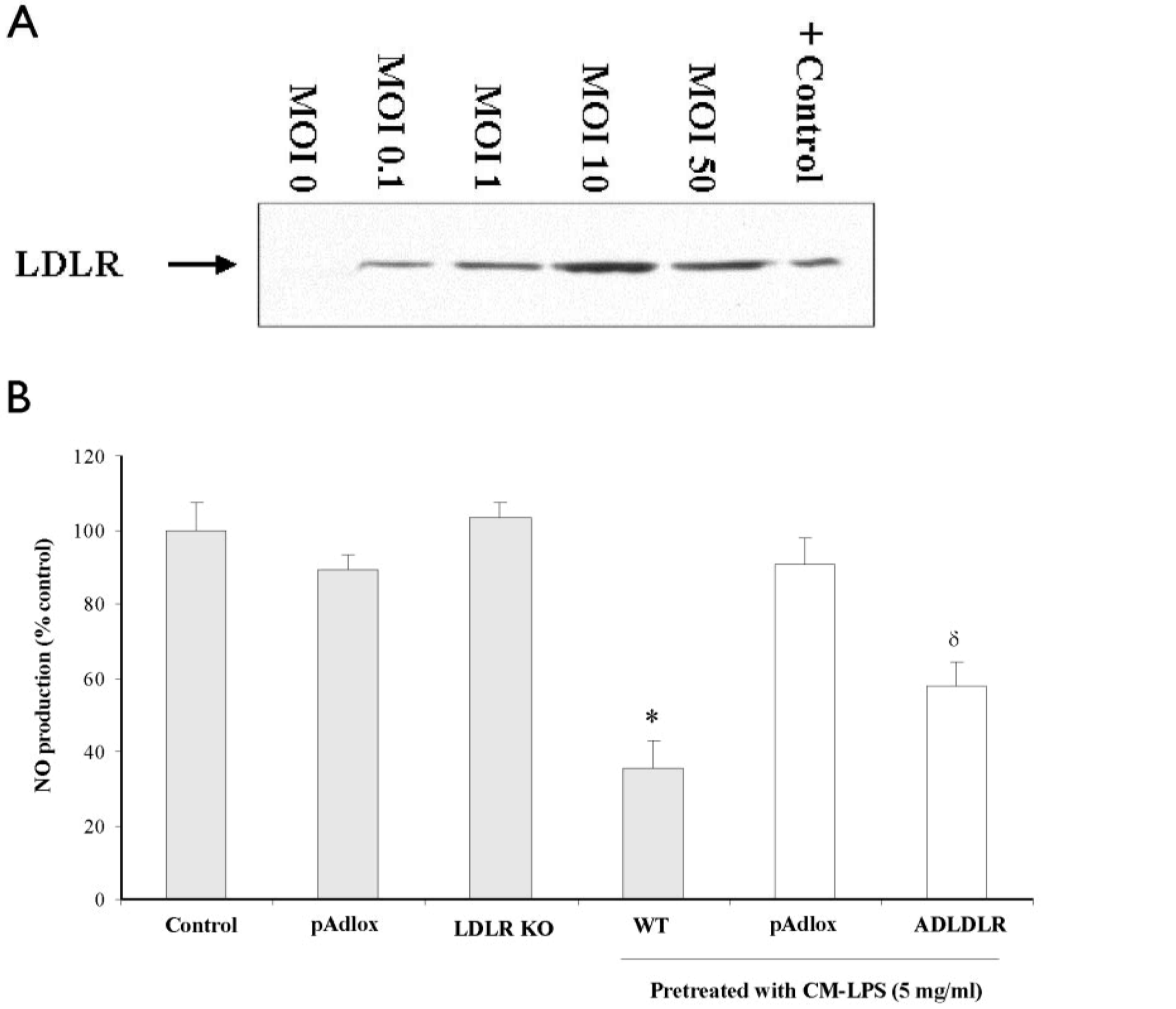

Fig. 5. Introduction of LDLR restores the response of *ldlr*$^{-/-}$ hepatocytes to sCM–LPS complexes. (A) Murine hepatocytes were infected with an adenovirus expressing the human LDLR (AdCMVhLDLR). Representative immunoblot demonstrating the expression of LDLR in *ldlr*$^{-/-}$ hepatocytes after infection at different MOIs. Extracts of wild-type rat hepatocyte membrane protein were used as a positive control (+ Control). (B) Effect of pretreatment on *ldlr*$^{-/-}$ hepatocytes after infection with AdCMVhLDLR. Cells in Ad-LDLR group were infected at a MOI of 10 and pretreated with sCM–LPS complexes for 2 h. After 16 h, they were stimulated with cytokines, and the rate of NO production was compared to the control group (uninfected, non-pretreated wild-type hepatocytes): WT, pretreated wild-type mouse hepatocytes; *ldlr*$^{-/-}$, uninfected, pretreated hepatocytes from LDLR-deficient mice; pAdlox, mouse hepatocytes infected with an adenovirus containing CFTR gene in antisense sequence. Data represent the mean ± SEM of 6 samples from two separate experiments, $^{*}P = 0.02$ compared to control; $^{\delta}P = 0.007$ compared to *ldlr*$^{-/-}$.

biochemical or genetic inhibition of the LDLR effectively inhibited the induction of cytokine tolerance, whereas restoration of LDLR function in LDLR-deficient hepatocytes restored the capacity for induction of the cytokine-tolerant phenotype.

This rapid attenuation of the hepatocellular response to cytokine stimulation using CM–LPS complexes implies that the process results from interference with cytokine signaling pathways, rather than via gene activation and/or *de novo* protein synthesis. Furthermore, the kinetics of cytokine tolerance suggest that a receptor-mediated process is responsible for its induction following pretreatment with CM–LPS complexes. Specifically, these findings indicate that the induction of cytokine tolerance in hepatocytes is primarily mediated by the LDLR, and that this receptor is the more important and physiologically relevant receptor for the expression of the tolerant phenotype.

We have shown that pretreatment with CM–LPS complexes induces a cytokine-tolerant phenotype in hepatocytes, which is time- and dose-dependent. The tolerance to pro-inflammatory cytokines also depends on functional lipoprotein receptors. However, the mechanism by which CM-bound LPS induces tolerance is unknown. Following internalization, chylomicrons move into the lysosomal compartment and are hydrolyzed. LPS bound

to the TG-rich lipoproteins is, therefore, most likely also delivered to lysosomes, where it is presumed to be degraded and detoxified. However, the presence of biologically active LPS in the bile and feces of rodents injected with radiolabeled LPS and the fact that CM increase the biliary excretion of LPS indicate that some fraction of the internalized LPS can escape lysosomal degradation.[18] Perhaps that population of LPS which has 'escaped' lysosomal degradation, either directly or indirectly, interferes with cytokine-signaling pathways in our model system.

Certainly, increased circulating triglyceride levels play a protective role in rodent models of endotoxemia, but the exact interrelationship between TG-rich lipoproteins, ApoE and the LDLR is not clear. As noted earlier, ApoE levels are inversely correlated with the susceptibility of rodents to LPS-induced toxicity. ApoE knockout mice show an increased mortality in response to LPS despite elevated levels of TG-rich lipoproteins in the circulation. However, LDLR-deficient animals are protected against LPS, seemingly in part due to the concomitant hyperlipoproteinemia. The immunodeficiency noted in ApoE knockout mice may help clarify these apparently discordant findings.[19–21] Nonetheless, cytokine tolerance is likely a means of negatively regulating the cytokine-mediated hepatic response to infection. As such, the TG-rich lipoprotein-mediated induction of cytokine tolerance can be interpreted as a component of the acute phase response, designed to help protect the host against invading bacterial pathogens. In contrast, cytokine tolerance may represent a mechanism by which Gram-negative bacteria serve to attenuate the innate immune response by using TG-rich lipoproteins as a Trojan-horse-like vehicle to enter hepatocytes. In this way, Gram-negative bacteria could effectively subvert the hepatocellular contribution to the host inflammatory response in an effort to evade destruction. Regardless of the teleology, these findings underscore an important interrelationship between TG-rich lipoprotein metabolism and innate immunity deserving of further investigation.

Acknowledgement

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