

## Free radical-mediated transgene inactivation of macrophages by endotoxin

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**Dokka, Sujatha, David Toledo, Liying Wang, Xianglin Shi, Chuanshu Huang, Stephen Leonard, and Yon Rojanasakul.** Free radical-mediated transgene inactivation of macrophages by endotoxin. *Am J Physiol Lung Cell Mol Physiol* 279: L878–L883, 2000.—Endotoxin, the lipopolysaccharide component of gram-negative bacteria, is a common contaminant of plasmid DNA preparations. The present study investigated the effect of endotoxin on gene transfection efficiency and the role of reactive oxygen species (ROS) in this process. Gene transfection studies were performed in various cell types with cytomegalovirus-luciferase as a reporter plasmid and cationic liposome as a transfecting agent. The presence of endotoxin in plasmid DNA preparations severely limited transgene expression in macrophages but had little or no effect in other cell types tested. This decreased transfection was dependent on ROS-mediated cellular toxicity induced by endotoxin. Neutralizing the endotoxin by the addition of polymyxin B effectively increased transfection efficiency and reduced toxicity. Electron spin resonance studies confirmed the formation of ROS in endotoxin-treated cells and their inhibition by free radical scavengers. The ROS scavenger *N*-*t*-butyl- $\alpha$ -phenylnitrone, the H<sub>2</sub>O<sub>2</sub> scavenger catalase, and the  $\cdot$ OH scavenger sodium formate effectively inhibited endotoxin-induced effects, whereas the O<sub>2</sub><sup>-</sup> scavenger superoxide dismutase had lesser effects. These results indicate that multiple oxidative species are involved in the transfection inactivation process and that  $\cdot$ OH formed by H<sub>2</sub>O<sub>2</sub>-dependent, metal-catalyzed Fenton reaction play a major role in this process.

gene transfection; free radicals; macrophages

MACROPHAGES PLAY AN IMPORTANT ROLE in host defense against noxious substances and are involved in a variety of disease processes including autoimmune diseases, infections, and inflammatory disorders (see Ref. 9 for review). Molecular analysis of macrophage functions can be accomplished by gene transfection assays. However, gene transfection in macrophages has proven difficult due to their refractory nature. Over the years, numerous efforts have been made to improve gene transfection of macrophages (2, 4, 10, 17), but their

transfection is still poor. Partly responsible for this limited success is a lack of understanding of the causes that contribute to the refractory nature of macrophages during gene transfection. Because macrophages are known to be highly susceptible to endotoxin stimulation and because endotoxin is a major contaminant of plasmid DNA preparations used in most transfection studies, we hypothesized that the presence of contaminating endotoxin may contribute to this low transfection. The present study was undertaken to test this hypothesis and to elucidate the mechanisms underlying the cellular unresponsiveness of macrophages to gene transfection.

Endotoxin [lipopolysaccharide (LPS)] is known to be an extremely bioactive substance and a potent stimulator of immune cells (13, 20). LPS consists of a variable polysaccharide domain covalently attached to a lipid domain (lipid A). LPS mediates most of its effects by binding to macrophages and inducing the production of many cellular mediators, including proinflammatory cytokines such as tumor necrosis factor- $\alpha$  and interleukin-1 (14), fatty acid metabolites (14), and reactive oxygen species (ROS) (8). ROS are known to exert multiple effects on cells and tissues and are involved in a variety of pathological processes. They can cause DNA damage, lipid peroxidation, protein modification, and activation of certain nuclear transcription factors such as activator protein-1 and nuclear factor- $\kappa$ B (11, 15). In the present study, we tested whether ROS are involved in the transfection inactivation process of macrophages.

LPS has been shown to reduce gene transfection efficiency in nonimmune cells (19); however, its mechanism of action is not known. We report here that cellular toxicity induced by LPS is responsible, at least in part, for the decreased transfection efficiency. Because macrophages are the primary cellular target for LPS stimulation, we postulated that this cell type may be more susceptible to LPS effects. We examined the role of ROS generation by macrophages in transfection

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efficiency and cytotoxicity. Our hypothesis is that if ROS are responsible for such effects, then blocking these reactive species, i.e., by free radical scavengers, would increase transfection efficiency and decrease cellular toxicity. We also attempted to identify key reactive species involved in the process. The following specific questions are addressed in this study. 1) Are macrophages more susceptible to LPS contamination of plasmid DNA than other cell types? 2) What is the underlying mechanism of LPS-induced cellular toxicity in macrophages? 3) Can free radical scavengers decrease this toxicity and do they reverse LPS-induced decrease in transfection? 4) If so, what are the key reactive species involved in the process?

## MATERIALS AND METHODS

**Cell culture.** All cell lines including RAW 264.7 and NR 383 macrophages, alveolar epithelial A549 cells, kidney embryonic 293 cells, and liver Hep G2 cells were obtained from the American Type Culture Collection (Manassas, VA). The cells were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 U/ml of penicillin-streptomycin. They were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Before use, the cells were briefly trypsinized or mechanically scraped and centrifuged. They were plated at  $\sim 1 \times 10^6$  cells/ml in 12-well tissue culture plates 1 day before the transfection studies.

**Plasmid DNA.** The expression vector cytomegalovirus (CMV)-luciferase contains the promoter-enhancer region of CMV upstream from the luciferase gene (kindly provided by Dr. Leaf Huang, University of Pittsburgh, Pittsburgh, PA). The plasmid was purified with QIAGEN EndoFree or regular ion-exchange plasmid kits (QIAGEN, Chatsworth, CA) according to the manufacturer's instructions. Content of the endotoxin in plasmid DNA preparations was determined by using the chromogenic *Limulus* amebocyte lysate assay (Kinetic-QCL, BioWhittaker, Walkersville, MD) according to the manufacturer's instructions. In studies designed to study the effect of endotoxin on gene transfer efficiency, specified amounts of LPS [*Escherichia coli* 0111:B4, 1 endotoxin unit (EU)/ $\mu$ g; Sigma, St. Louis, MO] were added to the plasmid preparations.

**Liposomal transfection.** Approximately  $1 \times 10^6$  cells were plated on 12-well plates and allowed to grow for 24 h before the transfection. Plasmid DNA (1  $\mu$ g/ml) was diluted in 200  $\mu$ l of DMEM (GIBCO BRL, Life Technologies), and the DNA-condensing agent protamine sulfate (0.1–2  $\mu$ g/ml; Sigma) was added to the DNA. Liposomes (1–20  $\mu$ g/ml) were diluted in 200  $\mu$ l of DMEM. The diluted DNA and liposome samples were combined and incubated at room temperature for 15–20 min. In some studies, indicated amounts of polymyxin B sulfate, *N*-*t*-butyl- $\alpha$ -phenylnitrone (PBN), superoxide dismutase (SOD), catalase, and sodium formate (Sigma) were also added to the transfection medium. The cells with the transfection reagents were incubated for 4 h. The transfection medium was then replaced with growth medium containing 10% fetal bovine serum. The cells were cultured for an additional 48 h before the level of gene expression was determined. All transfections were conducted under sterile conditions, and duplicate plates were tested for each condition.

**Measurement of luciferase activity.** Luciferase synthesized during the *in vitro* translation was quantitated with the assay of enzyme-dependent light production with a luciferase assay kit (Promega, Madison, WI). The cells were washed

twice with PBS, incubated at room temperature for 10 min in the presence of 250  $\mu$ l of lysis buffer (Promega), and then centrifuged at 12,000 *g*. Ten microliters of each sample were placed in a 5-ml polystyrene test tube, and the tubes were then loaded into an automated luminometer (Bio-Rad, Hercules, CA). At the time of measurement, 100  $\mu$ l of luciferase substrate were automatically injected into each sample, and total luminescence was measured over a 20-s time interval. Output was quantitated as relative light units. Protein content in the supernatant was determined by bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). Luminescence detected was standardized per microgram of protein present in the supernatant.

**Lactate dehydrogenase activity.** Lactate dehydrogenase (LDH) assay was performed to assess the effect of test agents on cellular toxicity. The cells were treated with plasmid DNA, LPS, and transfecting agents either individually or in combination as indicated. After the treatments, the cell supernatants were collected and assayed for LDH activity. LDH activity was determined by monitoring the oxidation of pyruvate coupled with the reduction of NAD at 340 nm with an LDH assay kit (Roche Diagnostic Systems, Montclair, NJ). The assay was performed on a Cobas Fara II analyzer (Roche Diagnostic Systems). One unit of LDH activity per liter is defined as the amount of enzyme that converts 1  $\mu$ mol of lactate to 1  $\mu$ mol of pyruvate, with the concomitant reduction of 1  $\mu$ mol of NAD to 1  $\mu$ mol of NADH per minute per liter of sample in the assay procedure.

**Free radical measurements.** The electron spin resonance (ESR) spin trapping technique was used to detect short-lived free radical intermediates. All measurements were conducted with a Varian E9 ESR spectrometer and a flat-cell assembly. Hyperfine splittings were measured (to 0.1 G) directly from magnetic field separations with potassium tetraperoxochromate (K<sub>3</sub>CrO<sub>8</sub>) and 1,1-diphenyl-2-picrylhydrazyl as standards. Reactants were mixed in test tubes in a total volume of 0.5 ml. The reaction mixture was then transferred to a flat cell for ESR measurement. All measurements were carried out with 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO; Aldrich) as a spin trap.

## RESULTS

**Macrophages are difficult to transfect.** To evaluate the relative transfection efficiency of macrophages compared with other cell types, we transfected various cell lines from different origins including RAW 264.7 and NR 383 macrophages, kidney embryonic 293 cells, alveolar epithelial A549 cells, and liver Hep G2 cells with the CMV-luciferase reporter plasmid. Gene transfection was carried out under the same transfection conditions with LipofectAMINE and protamine as transfecting agents. Optimum transfection conditions were determined, and transfection efficiencies between cell lines were compared. Figure 1 shows that maximum luciferase activity was observed in Hep G2 cells, followed by embryonic 293 cells, epithelial A549 cells, and RAW 264.7 and NR 383 macrophages. In the absence of transfecting agents, all five cell lines exhibited minimum luciferase activity. These results indicate that gene transfection is cell type dependent and that macrophages are relatively difficult to transfect compared with other cell types. All transfection studies were also carried out with a CMV- $\beta$ -galactosidase re-

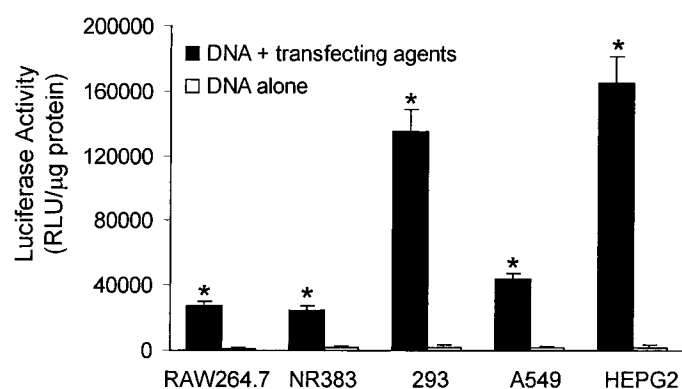


Fig. 1. Comparison of gene transfection in different cell lines. Cells ( $1 \times 10^6$ /ml) were transfected with the reporter plasmid cytomegalovirus (CMV)-luciferase (1  $\mu$ g/ml) in the presence and absence of transfecting agents (12  $\mu$ g/ml of LipofectAMINE and 1  $\mu$ g/ml of protamine) for 4 h at 37°C. The transfection conditions were optimized for maximum transfection of macrophages. Two days posttransfection, the cells were washed, lysed, and measured for luciferase activity (used to measure transfection efficiency). RAW 264.7 and NR 383, macrophages; 293, kidney embryonic cells; A549, alveolar epithelial cells; Hep G2, liver cells; RLU, relative light units. Data were normalized to protein content. Values are means  $\pm$  SD;  $n = 4$  experiments. \* $P < 0.05$  vs. free DNA control.

porter plasmid, and the results were consistent with those with CMV-luciferase.

**Endotoxin reduces transfection efficiency and increases cellular toxicity.** Endotoxin is known to be a frequent contaminant of plasmid DNA preparations (3) and a potent stimulator of macrophages (13, 20). We therefore tested whether the presence of contaminating endotoxin could contribute to the observed low transfection efficiency in macrophages. To test this possibility, we used highly purified plasmid preparations that we obtained by using the QIAGEN EndoFree plasmid preparation kit and tested the effect of added LPS on gene transfer efficiency. Plasmid samples prepared by this method were found to contain  $<0.1$  EU/ $\mu$ g plasmid DNA as determined by the *Limulus* amoebocyte lysate assay. This amount of endotoxin is typically 100–10,000 times less than that obtained by conventional methods of DNA preparation such as anion-exchange chromatography and silica-based adsorption (19). Not surprisingly, transfection of macrophages with the EndoFree plasmid was 30 times greater than that of plasmid prepared by anion-exchange chromatography (Fig. 2). Figure 3A shows that the addition of small amounts of LPS (0–0.5  $\mu$ g/ml or 0–5 EU/ml) greatly reduced the transfection efficiency of macrophages. In contrast, LPS at the same concentration range had no significant effect on gene transfection efficiency in other cell types tested (Fig. 3A). These results suggest that macrophages are especially sensitive to LPS and that this increased susceptibility may be responsible for their poor transfection efficiency. To test whether the reduced transfection is associated with cellular toxicity potentially caused by LPS, we studied the effect of LPS on cellular LDH release. Figure 3B shows that at the same concentrations used in gene transfection studies, LPS

caused a significant toxic effect in macrophages but had only minimal effect in other cell types. These results suggest that the low transfection efficiency in macrophages may be caused by LPS-mediated cellular toxicity.

The role of endotoxin in macrophage toxicity was further studied by using the endotoxin-neutralizing agent polymyxin B. Polymyxin B is a polycationic antibiotic that has been widely used to neutralize the effects of LPS (18). Treatment of the cells with this agent during gene transfection decreased cellular toxicity (Fig. 4A) and reversed the LPS-induced decrease in transfection (Fig. 4B). Thus these results confirm that the observed low transfection is due to the cytotoxic effects of endotoxin.

**ROS are involved in LPS-mediated toxicity.** LPS stimulation of macrophages has been reported to cause activation and release of ROS (8). Because ROS are known to be involved in a number of pathological processes, we postulated that ROS may be responsible for LPS-induced toxicity and reduced transfection. To test this possibility, we treated the cells with PBN (a ROS scavenger) and studied its effects on LPS-induced toxicity and transfection activity. PBN has been used in a previous study (7) as a scavenger of ROS. Our results show that PBN effectively inhibited LPS-induced toxicity (Fig. 4A) and restored transfection activity (Fig. 4B). Thus our results support the role of ROS in the process. Subsequent studies using specific ROS scavengers (see *Hydroxyl radical is the key reactive species responsible for LPS-induced effects*) further confirm these results.

**Hydroxyl radical is the key reactive species responsible for LPS-induced effects.** Because PBN is a nonspecific ROS scavenger, the identity of specific oxygen species involved in this process is not known. To identify such species, we used specific ROS inhibitors, including SOD ( $O_2^-$  scavenger), catalase ( $H_2O_2$  scavenger), and sodium formate [hydroxyl radical ( $\cdot OH$ ) scavenger] to study the effects on LPS.  $O_2^-$ ,  $H_2O_2$ , and  $\cdot OH$  are three major reactive species produced by mac-

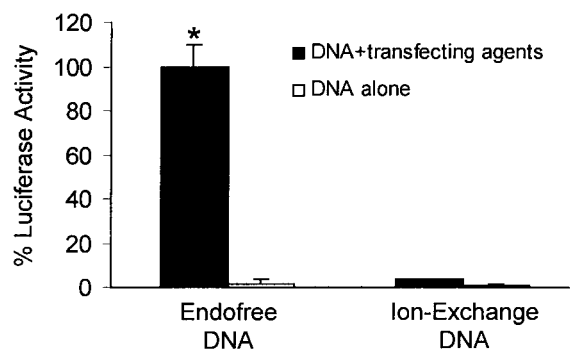


Fig. 2. Effect of DNA preparation on gene transfer efficiency. RAW 264.7 macrophages ( $1 \times 10^6$ /ml) were transfected with either EndoFree or anion-exchange plasmid CMV-luciferase (1  $\mu$ g/ml) in the presence and absence of transfecting agents (12  $\mu$ g/ml of LipofectAMINE and 1  $\mu$ g/ml of protamine) for 4 h at 37°C. Two days posttransfection, the cells were washed, lysed, and measured for luciferase activity. Values are means  $\pm$  SD;  $n = 4$  experiments. \* $P < 0.05$  vs. ion-exchange treatment.



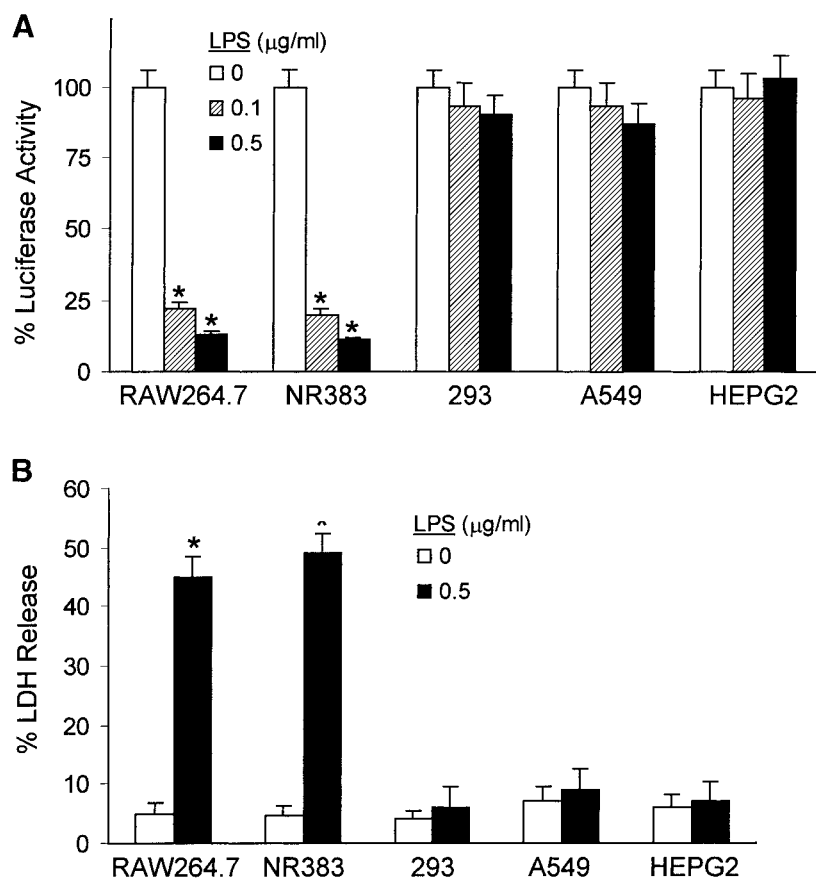


Fig. 3. Effects of endotoxin on transfection efficiency (A) and cellular toxicity [as measured by lactate dehydrogenase (LDH) release; B] in different cell lines. Cells ( $1 \times 10^6$ /ml) were transfected with EndoFree CMV-luciferase (1 µg/ml) in the presence of transfecting agents (12 µg/ml of LipofectAMINE and 1 µg/ml of protamine) for 4 h at 37°C. Indicated amounts of lipopolysaccharide (LPS) were also added to the transfection medium during transfection. Two days posttransfection, the cells and supernatants were collected and analyzed for luciferase and LDH activities. Values are means  $\pm$  SD;  $n = 4$  experiments. \* $P < 0.05$  vs. LPS-untreated control.

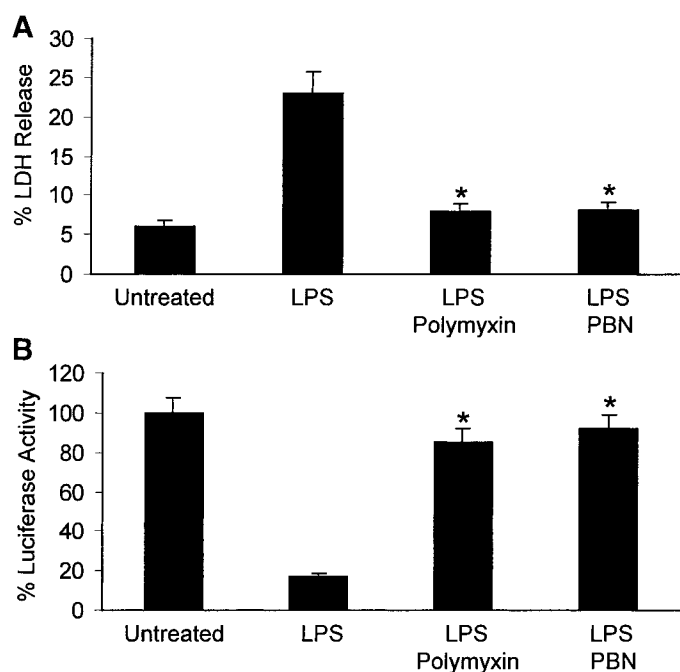


Fig. 4. Effects of polymyxin B and *N*-*t*-butyl- $\alpha$ -phenylnitrone (PBN) on LPS-induced toxicity (A) and transfection efficiency (B). Cells ( $1 \times 10^6$ /ml) were transfected with CMV-luciferase (1 µg/ml) in the presence of transfecting agents (12 µg/ml of LipofectAMINE and 1 µg/ml of protamine) for 4 h at 37°C. Polymyxin B (1 µg/ml) and PBN (2 mM) were added to the transfection medium in different treatment groups. Two days posttransfection, the cells and supernatants were collected and analyzed for luciferase and LDH activities. Values are means  $\pm$  SD;  $n = 4$  experiments. \* $P < 0.05$  vs. LPS-treated control.

rophages (5, 6, 16) and thus are the primary focus of our investigation. Figure 5, A and B, shows that all three scavengers had an inhibitory effect on cellular toxicity and transfection activity. However, the effects were more pronounced in the case of catalase and sodium formate, and lesser effects were observed with SOD. It should be noted that all three scavengers were tested at different concentrations; however, only optimal concentrations of each scavenger are presented here. The results obtained suggest that multiple ROS are involved in the toxicological process and that  $H_2O_2$  and  $\cdot OH$  play a greater role. Because  $H_2O_2$  has been reported to be a major source of  $\cdot OH$  formation in macrophages, i.e., via a metal-catalyzed Fenton reaction [ $M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + OH^- + \cdot OH$ ] (16), and because  $\cdot OH$  is known to be highly reactive, we suggest that  $\cdot OH$  may be the primary oxidative species responsible for the observed effects induced by LPS. Supporting this notion is the fact that sodium formate was equally as effective in decreasing toxicity and restoring transfection as catalase and that catalase can inhibit  $\cdot OH$  in macrophages as further demonstrated in our ESR studies (see below).

To confirm that  $\cdot OH$  was actually formed during LPS activation, ESR studies using the spin trap DMPO were carried out. Cells were treated with LPS in the presence and absence of ROS scavengers. Figure 6 shows that in the presence of LPS, an ESR spectrum consisting of a 1:2:2:1 quartet pattern, which is characteristic of a DMPO- $\cdot OH$  adduct (1), was observed.

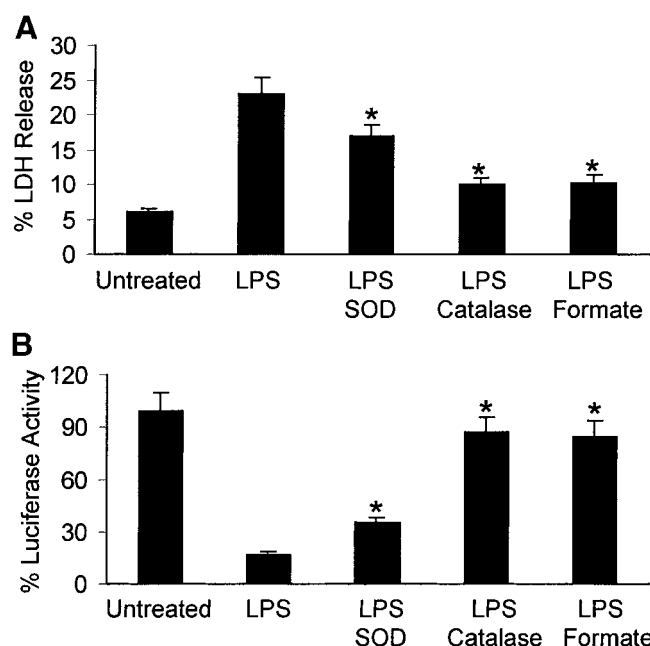


Fig. 5. Effects of reactive oxygen species (ROS) scavengers on LPS-induced toxicity (A) and transfection efficiency (B). Cells ( $1 \times 10^6$ /ml) were transfected with CMV-luciferase (1  $\mu$ g/ml) in the presence of transfecting agents (12  $\mu$ g/ml of LipofectAMINE and 1  $\mu$ g/ml of protamine) for 4 h at 37°C. Superoxide dismutase (SOD; 100  $\mu$ g/ml), catalase (100 U/ml), and sodium formate (5 mM) were added to the transfection medium in different treatment groups. Two days post-transfection, the cells and supernatants were collected and analyzed for LDH and luciferase activities. Values are means  $\pm$  SD;  $n = 4$  experiments. \* $P < 0.05$  vs. LPS-treated control.

Addition of the  $\cdot$ OH scavenger sodium formate to the system decreased the intensity of the DMPO- $\cdot$ OH signal, thus confirming the formation of  $\cdot$ OH induced by LPS. Interestingly, catalase, the function of which is to

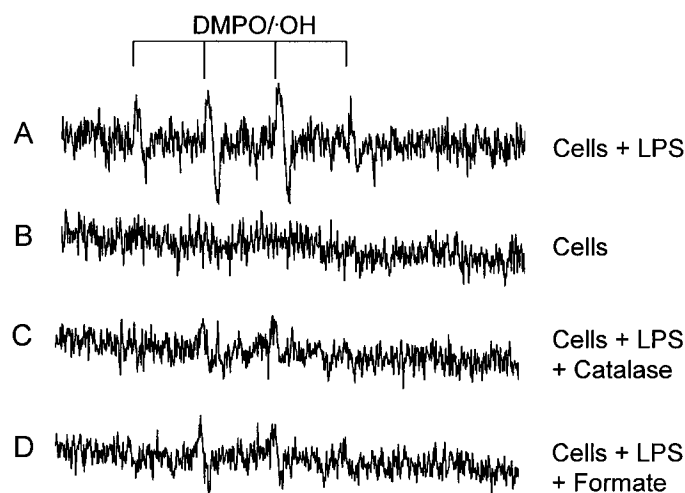


Fig. 6. Electron spin resonance (ESR) measurements of LPS-induced ROS generation. A: ESR spectrum recorded 1 h after an addition of LPS (10  $\mu$ g/ml) to RAW 264.7 macrophages ( $1 \times 10^6$ /ml) in the presence of the spin trapper 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO; 100 mM). B: same as A but with no LPS treatment. C: same as A but with catalase (100 U/ml) added. D: same as A but with sodium formate (5 mM) added. The spectrometer settings were as follows: receiver gain,  $1.5 \times 10^5$ ; time constant, 0.3 s; modulation amplitude, 1.0 G; scan time, 4 min; and magnetic field,  $3,470 \pm 100$  G.

scavenge  $H_2O_2$ , also inhibited  $\cdot$ OH formation. These results are consistent with a previous study by Rojanasakul et al. (16) that demonstrated that  $H_2O_2$  can react with endogenous metal ions to form  $\cdot$ OH via the Fenton reaction. The results also explain the observed comparable effects of catalase and sodium formate in our toxicity and transfection studies.

## DISCUSSION

Transfection of macrophages represents a significant challenge in the gene regulation studies that utilize these cells. Because of their crucial function in a variety of biological processes and pathologies, these cells also represent important targets for gene therapies. Using a liposome-based gene transfection assay, we have shown that macrophages are difficult to transfect compared with other cell types. Macrophages are sensitive to the content of contaminating endotoxin and hence are sensitive to the method of DNA preparation.

A previous study (19) has shown that the presence of contaminating endotoxin in plasmid preparations can reduce gene transfection efficiency in other cell types. However, this effect is generally observed at high levels of endotoxin, i.e.,  $>100$  EU/ml. In this study, we found that macrophages are susceptible to endotoxin at very low levels (0–5 EU/ml). At these concentrations, gene transfection was greatly diminished in macrophages but was relatively unaffected in other cell types. These results indicated that macrophages are particularly sensitive to endotoxin contamination and that this increased susceptibility may be responsible for their poor transfection efficiency.

The mechanism by which endotoxin decreases gene transfection in macrophages is not known. We suggest that this decreased efficiency may be associated with cellular toxicity induced by endotoxin. Supporting this notion is evidence that endotoxin induced cellular toxicity and also decreased gene transfection in macrophages, effects that were not observed in other cell types tested. The role of cellular toxicity in decreasing transfection efficiency is confirmed by treatment of the cells with polymyxin B during transfection. Polymyxin B is a polycationic antibiotic that has been widely used to neutralize the effects of LPS. Polymyxin B is known to bind the lipid A portion of LPS with high affinity (12). The lipid A portion has also been shown to be responsible for most of the biological activities of LPS (13, 14). We have observed in this study that the addition of polymyxin B to the transfection medium effectively inhibited the cytotoxic effect of endotoxin and restored the gene transfection efficiency of macrophages.

Endotoxin is known to activate macrophages and induce the production of various cellular mediators including ROS (8, 14). Consistent with these studies, our ESR and ROS scavenging studies indicated the formation of ROS in our system. To test whether the ROS generated were responsible for the decreased transfection induced by LPS, we treated the cells with different ROS scavengers during transfection. All scavengers increased gene transfection efficiency, thus sup-

porting the role of ROS in this process. These scavengers also decreased cellular toxicity induced by LPS, further substantiating the relationship between these two processes. The observation that all ROS scavengers exhibited LPS-inhibitory effects and that PBN, a general ROS scavenger, was more effective than other scavengers in neutralizing the LPS effects also indicated that multiple ROS are involved in the process. Careful analysis of the test results further showed that  $O_2^-$  plays a less significant role and  $\cdot OH$  formed by a  $H_2O_2$ -dependent, metal-catalyzed Fenton reaction plays a major role in the process.

The conclusions of this study are that 1) LPS decreases transfection efficiency in macrophages due to its toxic effect, 2) LPS-induced ROS generation is involved in this process, 3) inactivating LPS by the addition of polymyxin B or the addition of ROS scavengers decreases the toxicity associated with LPS, and 4)  $\cdot OH$  appears to be the major reactive species responsible for LPS-induced toxicity and reduced transfection. Several possibilities exist with regard to the effect of ROS on transfection activity. Cellular toxicities included by ROS would impair endocytic activity of the cells and hence transfection activity. Other possibilities include DNA damage and alterations in liposome binding and fusion activities.

We report here, for the first time, the role of ROS in causing decreased transfection in macrophages. Gene transfer studies of the lung are crucial to the understanding of normal and pathological lung functions at a molecular level. The results of this study should facilitate further mechanistic studies of lung cell physiology and pathology when gene transfer methodology is utilized.

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