Geldanamycin Treatment Ameliorates the Response to LPS in Murine Macrophages by Decreasing CD14 Surface Expression

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Submitted August 14, 2002; Revised October 3, 2002; Accepted October 25, 2002
Monitoring Editor: Keith R. Yamamoto

Geldanamycin (GA) is an antibiotic produced by *Actinomyces*, which specifically inhibits the function of the heat shock protein 90 family. Treatment of a murine macrophage cell line (J774) with GA resulted in a reduced response to *Escherichia coli* lipopolysaccharide (LPS) as visualized by a decrease of NF- κ B translocation into the nucleus and secretion of tumor necrosis factor α (TNF- α). To elucidate the mechanism of this effect, the expression of CD14, the formal LPS receptor, was analyzed. Cells treated with GA showed a reduced level of surface CD14 detected by immunostaining, whereas the expression of other surface receptors, such as FC- γ receptor and tumor necrosis factor receptors (TNF-R1 and TNF-R2), was unaffected. The reduced surface level of CD14 was not due to a reduction in its expression because CD14 steady state mRNA levels or the total cellular pool of CD14 was not altered by GA treatment. Surface CD14 was more rapidly internalized after GA treatment (2–3 h) than after incubation with cycloheximide. Immunostaining of permeabilized cells after GA treatment revealed a higher intracellular content of CD14 colocalizing with calnexin, an endoplasmic reticulum (ER) protein. These results suggest that the decrease in CD14 surface expression after GA treatment is due to rapid internalization without new replacement. These effects may be due to the inhibition of Hsp90 and Grp94 by GA in macrophages.

INTRODUCTION

Sepsis is a major complication observed in patients admitted to the surgical intensive care units after trauma, burn injury, pancreatitis, and even major surgery. Approximately 250,000 cases per year are observed within the United States (Angus and Wax, 2001). The presence of bacterial lipopolysaccharide (LPS) is an important factor in the incidence of sepsis. LPS is a component of the outer membrane of Gram-negative bacteria that is shed into circulation during infection. LPS triggers an inflammatory response directed to combat the pathogens. If this inflammatory process is not properly controlled, it may initiate a hypermetabolic condition termed endotoxic shock. Endotoxic shock is likely to proceed to the sequential collapse of different organ systems, coined multiple organ dysfunction syndrome. This syndrome is the major cause of morbidity and mortality in patients admitted into the surgical intensive care units (Meakins, 1990).

Administration of LPS to human volunteers and other animal species mimics many symptoms observed in sepsis. The first target of LPS is a 60-kDa glycoprotein, named LPS-binding protein (LBP), which is present in circulation during normal conditions. The complex between LPS and LBP is recognized by CD14, a cell surface receptor. CD14 is a glycosylphosphatidylinositol (GPI)-anchored membrane receptor expressed predominantly on the surface of myeloid cells (Tobias *et al.*; 1995). The GPI domain of CD14 acts as a membrane attachment structure and sorting signal (Lisanti *et al.*, 1990). Several proteins, which have been identified to contain a GPI modification, play an important role in leukocyte and macrophage function during inflammation (Zhang *et al.*, 1991). GPI-anchored proteins are present in membrane domains rich in cholesterol and sphingolipids, named lipid rafts (Robinson, 1991). The lack of transmembrane and cytosolic domains in CD14 does not allow this molecule to transduce a signal inside the cell. Thus, a family of transmembrane proteins named toll-like receptors has been identified as the signal transduction components in the response to LPS (Poltorak *et al.*, 1998; Jiang *et al.*, 2000). The LPS signal transduction pathway is complex, involving the activation of several protein kinases (i.e., ERKs, JNKs, and p³⁸ kinases), and transcriptional factors (i.e., NF- κ B and

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E02-08-0498. Article and publication date are at www.molbiolcell.org/cgi/doi/10.1091/mbc.E02-08-0498.

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AP-1), followed by synthesis and release of inflammatory mediators, such as cytokines, reactive oxygen species, and lipid mediators (Mackman *et al.*, 1991; Vincenti *et al.*, 1992; Sanlioglu *et al.*, 2001).

Geldanamycin (GA) is a benzoquinone ansamycin isolated from *Actinomyces* that was originally described as a tyrosine kinase inhibitor (DeBoer *et al.*, 1970). Subsequent studies have demonstrated that GA binds exclusively and with high affinity to the ATP binding site of the heat shock protein (Hsp) 90 family, interfering with their function (Whitesell *et al.*, 1994; Chavany *et al.*, 1996). Treatment of human respiratory epithelial cells and a macrophage cell line (RAW 264.7) with GA resulted in a reduced LPS response as indicated by a decrease of TNF- α production (Byrd *et al.*, 1997; Malhotra *et al.*, 2001). This effect was correlated with a decrease in binding of DNA by NF- κ B, suggesting that the lower response to LPS was due to blunting the mechanisms responsible for the production of proinflammatory mediators (Malhotra *et al.*, 2001). In the present study, we found that the effect of GA in macrophages is more upstream than the inhibition of NF- κ B activation, and it is related to the disappearance of CD14 from the cell surface. Thus, cells lacking the main receptor for LPS are incapable of responding to this important inflammatory mediator. In addition, we found that GA treatment results in the arrest of CD14 within the endoplasmic reticulum (ER), perhaps related to the improper folding of this glycoprotein.

MATERIALS AND METHODS

Reagents

Lipopolysaccharide (LPS) from *Escherichia coli* (026:B6) was obtained from Difco Laboratories (Detroit, MI). Geldanamycin from *Streptomyces hygroscopicus* was from Sigma Chemical Co. (St. Louis, MO). Anti-mouse CD14 conjugated with fluorescein isothiocyanate (CD14-FITC) antibody was purchased from PharMingen International (San Diego, CA). Anti-mouse calnexin, anti-mouse Hsp90, anti-mouse Grp94, and anti-mouse Hsp70 antibodies were purchased from StressGen Biotechnologies Corp. (Victoria, British Columbia, Canada). Antibody against mouse CD14, anti-mouse NF- κ B, anti-I κ B α , and anti-goat IgG conjugated with HRP were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-rabbit IgG antibody conjugated with Cy³ was purchased from Sigma Chemical Co.

Cell Culture Conditions and Treatment Conditions

J774A.1 cells were purchased from American Type Culture Collection (Rockville, MD). J774 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. LPS (100 ng/ml) stimulation was made at different time points up to 5 h. Cells were treated with GA (1 μ g/ml) and stimulated with LPS (100 ng/ml) in the presence of the drug. Cells were treated with cycloheximide (1 mg/ml) for 2–6 h. TNF- α in the extracellular medium was measured by a commercial ELISA (BioSource, Camarillo, CA).

Western Blotting

For CD14 detection, cells were washed twice with cold phosphate-buffered saline (pH 7.4) and scraped in the presence of 1 ml of 10 mM Tris-HCl, pH 7.4, 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 4 mg/ml trypsin inhibitor, 1 mg/ml benzamide, 5 μ M leupeptin, 200 μ M sodium vanadate, 100 nM okadaic acid, and 1 mg/ml PMSF. Cell suspensions were sonicated on ice (0.3 A, 10 s), and protein concentration was determined by the BCA

method (Pierce Chemical, Rockford, IL). Total proteins (100 μ g) were separated by SDS-PAGE (10% polyacrylamide gels) and transferred onto a nitrocellulose membrane (400 mA for 3 h). Membranes were blocked with 30% human serum for 45 min at 4°C, followed by 5% nonfat milk, 3% BSA in TBS, pH 7.4, for 1 h at 4°C. Blots were incubated with anti-mouse CD14 antibody (1:5000 dilution) in TBS for 16 h at 4°C. Membranes were rinsed twice with washing solution (0.3% Tween-20, 0.05% NP-40 in TBS, pH 7.4), and incubated with anti-goat IgG antibody conjugated to HRP (1:40,000) in TBS for 2 h at 4°C. Visualization of the bound complex was made by the chemiluminescent Super Signal kit (Pierce). For the detection of Hsp90, Grp94, and Hsp70, cells were lysed in 1 ml of 50 mM Tris-HCl containing 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 4 mg/ml trypsin inhibitor, 1 mg/ml benzamide, 5 μ M leupeptin, 200 μ M sodium vanadate, 100 nM okadaic acid, 1 mg/ml PMSF, 150 mM NaCl, and 1% Triton X-100. Blots were incubated with anti-mouse Hsp70, Grp94, or Hsp90 antibodies (1:5000 dilution), followed by secondary antibodies coupled to HRP and detected as described above.

Northern Blotting

Total RNA was isolated by the Trizol reagent (Life Technologies, Rockville, MD). RNA (10 μ g) was separated in formaldehyde-agarose gels and transferred onto a nylon modified membrane (Gene Screen Plus). Blots were hybridized at 42°C with radiolabeled cDNA probes using the random primer method (Feinberg and Vogelstein, 1983) with [α -³²P]dATP and [α -³²P]dCTP as previously described (Beck and De Maio, 1994). Blots were washed at 42°C two times with 6 $\times$ SSC, 1% SDS followed by two washes with 2 $\times$ SSC, 0.1% SDS. Blots were exposed to Molecular Dynamics Phosphor Screen System (Sunnyvale, CA) for 3 h. Signals were normalized to the intensity of 18S rRNA as determined by prestaining of the membranes with ethidium bromide.

Immunostaining

Cells on glass cover slides (0.5–1.0 $\times$ 10⁶ cells/slide) were incubated with or without GA (1 μ g/ml) for 16 h at 37°C, followed by any additional treatment as indicated in the figure legends. Cells were fixed in 4% paraformaldehyde (PFA) solution and permeabilized, when indicated, with acetone (15 s, 4°C). Nonspecific binding was blocked by incubation with 1% human serum (60 min, 4°C). Fixed cells were incubated with primary antibodies (1:200) for 60 min at 4°C, washed extensively with PBS, and incubated with secondary antibodies conjugated with Cy³ (1:1000) for 30 min at 4°C. Cells were further washed with PBS and mounted, and antibody binding was visualized using a fluorescence microscope. Nuclei were detected by DAPI staining.

RESULTS

Geldanamycin Treatment Decreases the Synthesis of LPS-induced TNF- α

J774 cells were incubated in presence or absence of GA (1 μ g/ml) for 16 h. Then, LPS (100 ng/ml) was added to the cells, which were further incubated for 1–5 h. At the end of the incubation period, the extracellular medium was collected, and TNF- α levels were measured by ELISA. Incubation with LPS resulted in a time-dependent increase in extracellular levels of TNF- α in absence of GA treatment. LPS-induced TNF- α levels were significantly reduced in cells treated with GA (Figure 1A). To further substantiate these findings, steady state TNF- α mRNA levels were evaluated in cells treated or not with GA and stimulated with LPS. Lower LPS-induced TNF- α mRNA levels were detected in cells treated with GA compared with cells that were not incubated with this drug (Figure

Figure 1. LPS-induced TNF- α expression is reduced in the J774 macrophage line by GA treatment. (A). Cells (2.5×10^5) were incubated with or without GA ($1 \mu\text{g/ml}$) for 16 h and stimulated with LPS (100 ng/ml) from 1–5 h. TNF- α levels were measured in the extracellular medium by ELISA and normalized by the number of viable cells in each well measured by the MTT assay (pg/ml/OD_{540 nm}). (B) Cells (6×10^6) were incubated with or without GA ($1 \mu\text{g/ml}$) for 16 h, stimulated with LPS (100 ng/ml) from 1 to 5 h and lysed for RNA extraction. Total RNA was analyzed by Northern blotting using a radiolabeled TNF- α cDNA probe. The signal was quantitated using Chemi-Genius Imaging system (Syngene) and normalized to the intensity of 18S rRNA determined by prior ethidium bromide staining of the membrane. Results are expressed as arbitrary units (A. U.). Statistical analysis for A and B was performed by one way analysis of variance (ANOVA) followed by Student's *t* test **p* < 0.05 with respect to untreated cells.

1B). These observations suggest that treatment with GA resulted in a reduced expression of TNF- α . GA treatment did not alter cell viability during the incubation time even in the presence of LPS, as demonstrated by the MTT assay (unpublished data).

LPS-dependent Translocation of NF- κ B into the Nucleus Is Impaired in GA-treated Cells

The synthesis of TNF- α is mediated by the activation and translocation into the nucleus of the transcriptional factor NF- κ B. Cells were cultured on cover slides in presence or

Figure 2. GA treatment blocks 78 F- κ B nuclear translocation and I κ B α degradation after incubation with LPS. Cells (0.5×10^6) on a glass cover slide were incubated with GA (D–F) or without the drug (A–C) for 16 h. Cells were stimulated with LPS (100 ng/ml) for 25 min. in presence (J–L) or absence (G–I) of GA. Cells were fixed with PFA, permeabilized with acetone and immunostained for NF- κ B (A, D, G, and J) or I κ B α (B, E, H, and K). Figures C, F, I, and L correspond to the superposition of NF- κ B and I κ B α images. NF- κ B and I κ B α detection was performed using secondary antibodies conjugated with FITC and Cy³, respectively. Nuclei were stained with DAPI.

absence of GA ($1 \mu\text{g/ml}$) for 16 h and further incubated with LPS (100 ng/ml) for 25 min. Cells were fixed as described in MATERIALS AND METHODS and immunostained with antibodies against I κ B α and NF- κ B. Treatment with GA did not affect the cellular distribution of NF- κ B (Figure 2D) or I κ B α (Figure 2E). Incubation with LPS resulted in the translocation of NF- κ B into the nucleus and the degradation of I κ B α in absence of GA treatment (Figure 2, G and H, respectively). These two events were not observed in cells treated with GA and stimulated with LPS (Figure 2, J and K). These data support the hypothesis that the pathway involved in TNF- α expression is affected by GA treatment.

Figure 3. GA treatment decreases cell surface expression of CD14. Cells (5×10^5) on a glass cover slide were incubated (B) or not (A) with GA ($1 \mu\text{g}/\text{ml}$) for 16 h. Cells were fixed with PFA and immunostaining was performed using FITC-conjugated anti-mouse CD14 antibody. Nuclei were staining with DAPI.

Geldanamycin Treatment Reduces Cell Surface Expression of CD14

In an attempt to elucidate the mechanism through which GA decreases TNF- α expression in J774 macrophages, surface levels of CD14 were evaluated. CD14 is the major receptor target for LPS (Han *et al.*, 1993; Haziot *et al.*, 1996). Cells were cultured on glass cover slides and incubated with GA for 16 h, fixed, and evaluated for the presence of surface CD14 by immunostaining. A dramatic reduction in plasma membrane CD14 was observed in GA-treated cells (Figure 3B), compared with untreated cells that showed a typical cell surface staining for this glycoprotein (Figure 3A). Stimulation of J774 cells with LPS (3 h) resulted in a significant

Figure 4. GA treatment does not affect protein or mRNA levels of CD14. Cells (6×10^6) were incubated with or without GA ($1 \mu\text{g}/\text{ml}$) for 16 h and incubated with LPS ($100 \text{ ng}/\text{ml}$) for 3–5 h. Then, cells were lysed for protein analysis or for RNA extraction. (A) Western blot for CD14. Cell extracts ($100 \mu\text{g}$ of protein) were separated by SDS-PAGE, transferred onto a nitrocellulose membrane and immunodetected using HRP-conjugated anti-CD14 antibody. (B) Northern blot for CD14 mRNA. Total RNA ($10 \mu\text{g}$) was separated in a formaldehyde-agarose gel, transferred onto nylon modified membrane and hybridized with a radiolabeled cDNA probe for CD14. These blots are representative of three independent experiments.

increase of cell surface CD14 in absence of GA treatment that was not observed in GA-treated cells (unpublished data). These observations suggest that the lack of response to LPS in GA-treated cells is probably due to the absence of cell surface CD14. To evaluate whether the reduction of CD14 from the cell surface in GA-treated cells was exclusive for this glycoprotein, the presence of other cell surface molecules was analyzed. Surface expression of Fc γ receptor and tumor necrosis factor receptors (TNF-R1 and TNF-R2) was not affected by treatment with GA (unpublished data), indicating that the effect of GA is not an overall reduction of cell surface proteins.

Geldanamycin Does Not Alter the Total Amount of CD14 or the Encoding mRNA

The total cellular pool of CD14 was evaluated in GA-treated J774 cells by Western blotting. Cells were incubated with or without GA ($1 \mu\text{g}/\text{ml}$) for 16 h and stimulated with LPS ($100 \text{ ng}/\text{ml}$) for 3–5 h in the presence of GA, and cellular extracts were prepared by sonication. Western blot analysis showed no differences in total CD14 content in cells treated or not with GA, even in the presence of LPS (Figure 4A). In agreement with this observation, CD14 mRNA levels detected by Northern blotting were similar between cells treated or not with GA (Figure 4B).

Figure 5. GA treatment results in a rapid disappearance of CD14 from the cell surface. Cells (5×10^5) were incubated with GA ($1 \mu\text{g}/\text{ml}$) for 2, 3, 4, 5, and 6 h (B, C, D, E, and F, respectively) or without the drug (A), fixed with PFA and CD14 visualized by immunostaining using an anti mouse-FITC conjugated antibody.

CD14 Disappears Rapidly from the Cell Surface upon Treatment with GA

J774 cells were incubated with GA ($1 \mu\text{g}/\text{ml}$) for 2–6 h, and surface CD14 was monitored by immunostaining. CD14 was observed to disappear from the cell surface rapidly during GA treatment. Approximately half of CD14 cell surface staining was reduced between 2 and 3 h of this treatment (Figure 5). A similar experiment was performed in J744 cells treated with cycloheximide ($1 \text{ mg}/\text{ml}$) to block new protein synthesis. In this case, CD14 also disappeared from the cell surface. However, the kinetics of this process was much slower than that observed in cells treated with GA (Figure 6). Cells were also coincubated with GA and cycloheximide and the presence of surface CD14 was monitored. The kinetics of CD14 disappearance from the cell surface was not different between coincubation with both drugs and GA alone (unpublished data). This result suggests that internalization of CD14 after GA treatment does not require new protein synthesis.

CD14 Is Retained in the ER after Treatment with GA

Immunostaining of permeabilized cells with antibodies against CD14 revealed that this protein is localized around the nucleus in GA-treated cells (Figure 7D). This observation is in contrast with untreated cells that showed the majority of CD14 on the cell surface (Figure 7A). This distribution of CD14 in GA-treated cells suggests a possible ER localization. To test this hypothesis, colocalization of CD14 and calnexin, an ER resident protein, was evaluated by immunostaining. Both CD14 and calnexin were observed in the same subcellular compartment in GA-treated cells (Figure 7F), suggesting that CD14 accumulates within the ER in GA-treated cells. There was little colocalization of CD14 and calnexin in absence of GA treatment (Figure 7C). Accumulation of CD14 within the ER could be observed after 8 h of GA treatment (unpublished data). Other plasma membrane proteins, such as Fc γ receptor and TNF-R1, and connexin 43 did not accumulate within the ER after GA treatment (unpublished data).

Figure 6. Cycloheximide treatment reduces surface expression of CD14. Cells (5×10^5) were incubated with cycloheximide (1 mg/ml) for 2, 3, 4, 5 and 6 h (B, C, D, E, and F, respectively) or without the drug (A), fixed with PFA and CD14 visualized by immunostaining using an anti-mouse FITC-conjugated antibody.

Geldanamycin Treatment Alters Heat Shock Protein Expression

Because GA is an inhibitor of the Hsp90 family, the effect of this drug on the expression of Hsps was evaluated. A reduction of Hsp90 and Grp94 levels was observed by Western blotting after 16 h of GA treatment (Figure 8, A and B), which was not modified by LPS stimulation. On the contrary, Hsp70 expression was induced by GA treatment, which appears to be enhanced by LPS stimulation (Figure 8C). The decrease in the detection of Hsp90 and Grp94 could be due to the fact that GA affects the conformation of these proteins, modifying the recognition by the antibody used in this study, which has been reported to be partially maintained after Western blotting (Melnick *et al.*, 1992).

DISCUSSION

Macrophages play a central role in the innate immune response to microbial pathogens. LPS, a major constituent of the outer membrane of Gram-negative bacteria, is a potent activator of macrophages both in vivo and in vitro (Auger

and Ross, 1992). The first step of this process is LPS binding to LBP and their association with CD14 on the surface of macrophages (Tobias *et al.*, 1995). Functional interaction of this ternary complex with toll-like receptor 4 leads to the activation of the inflammatory process, which is characterized by the expression of several mediators, including TNF- α (Jiang *et al.* 1995; Chavany *et al.* 1996; Malhotra *et al.*, 2001). In the present study, we showed that treatment of a murine macrophage cell line (J774) with GA, an inhibitor of the Hsp90 family, resulted in a decrease in NF- κ B translocation into the nucleus and the subsequent reduction of TNF- α production. This observation is consistent with prior observations using this drug in the macrophage cell line RAW 264.7 (Byrd *et al.* 1997) and human respiratory epithelial cells (Malhotra *et al.*, 2001). Moreover, we demonstrate that the underlying mechanism in this reduced response to LPS in GA-treated J774 cells is related to a reduction of CD14 on the cell surface.

The effect of GA on CD14 expression seems to occur at different levels: accelerated internalization of CD14 from the plasma membrane and accumulation of CD14 within the ER

Figure 7. GA treatment results in the arrest of CD14 within the ER. Cells (5×10^5) were incubated with GA (D, E and F) or without (A, B, and C) for 16 h, fixed with PFA and permeabilized with cold acetone. CD14 was detected using an anti-mouse antibody conjugated to FITC (A and D). Calnexin was detected by an anti-mouse antibody followed by a Cy³-conjugated secondary antibody (B and E). Nuclei were stained with DAPI. (C and F) The combination of these images.

after GA treatment. These observations raise several questions. One of them is why CD14 is arrested within the ER in GA-treated cells. A possibility is that CD14 is retained within the ER because it is not properly folded. Grp94 is an ER resident chaperone that is involved in the folding of several proteins (Wearsch and Nichitta, 1997; Nicchitta, 1998). Thus, it is likely that the inhibition of Grp94 function by GA resulted in the incomplete folding of proteins within the ER. These unfolded proteins may be retained in the ER as part of the quality control system that is present in this subcellular compartment (Gething and Sambrook, 1992). Lawson *et al.* (1998) have suggested that the inhibition of Grp94 by GA produces an alteration in the folding and/or assembly of nascent proteins. GA has also been reported to disrupt complexes between Grp94 and erbB2, causing the degradation of the latter protein by the ubiquitin/proteasome pathway (Chavany *et al.*, 1996; Xu

et al., 2001). Grp94 has also been proposed to be involved in the folding, assembly and export of toll like receptors, integrins, and immunoglobulin (Schaiff *et al.*, 1992; Ferreira *et al.*, 1994). The interaction between Grp94 and target proteins has been difficult to detect (Schaiff *et al.*, 1992; Ferreira *et al.*, 1994; Chavany *et al.*, 1996). Nevertheless, Grp94 has been found to be associated with Ig chains (Ferreira *et al.*, 1994), MHC class II (Schaiff *et al.*, 1992), thyroglobulin (Kuznetsov *et al.*, 1994), erbB2 (Chavany *et al.*, 1996), a herpes virus glycoprotein (Ramakrishnan *et al.*, 1995), collagen (Ferreira *et al.*, 1994), and apolipoprotein B (Linnik and Herscovitz, 1998). Grp94 cooperates with other ER chaperones, such as Grp78 (BIP), calreticulin, calnexin, protein disulfide isomerase, and Hsp74, in the process of protein folding (Tatu and Helenius, 1997). Although we proposed that the retention of CD14 within the ER is due to inhibition of Grp94 by GA, we cannot

Figure 8. Geldanamycin treatment alters heat shock protein expression. Cells (6×10^6) were cultured with GA ($1 \mu\text{g/ml}$) for 16 h. Cells were stimulated with LPS (100 ng/ml) for 3–5 h and lysed. Lysates ($100 \mu\text{g}$ of protein) were analyzed by Western blotting using antibodies against Hsp90 (A), Grp94 (B), and Hsp70 (C). These blots are representative of three independent experiments.

discard the possibility that the cytosolic chaperone Hsp90 may be involved in the processing of CD14 because GA also binds to Hsp90 (Whitese *et al.*, 1994). This latter possibility is less likely because CD14 lacks a cytosolic tail. Another option is that GA treatment interferes with the GPI anchoring of CD14. GPI is synthesized as a block by sequential additions of sugars and ethanolamine phosphates to phosphatidylinositol within the ER (Sharma *et al.*, 1999). The moiety is then added to a specific domain within the C-terminal of the target proteins (Simons and Ikonen, 1997). GPI anchored proteins are localized within cholesterol and sphingolipid-rich membrane microdomains, termed lipid rafts (Brown and Rose, 1992). One important characteristic of proteins in lipid raft is their inability to be solubilized by nonionic detergents (Brown *et al.*, 1992). Thus, it is possible that Grp94 is necessary for the retention of CD14 within the ER to guarantee addition of the GPI moiety. This possibility is also unlikely because we have not been able to solubilize any CD14 with non-ionic detergents in cells treated with GA.

In addition to the retention of CD14 within the ER, a rapid internalization of this glycoprotein was also observed in cells treated with GA. This effect seems to be independent from the export of CD14 to the plasma membrane. Under normal circumstances, CD14 is present in

internal vesicles that fused with the plasma membrane upon activation with LPS (Detmers *et al.*, 1995). Moreover, CD14 is a glycoprotein with a short half-life, which has been estimated to be 3 h. Thus, it is expected that the turnover of this glycoprotein from the cell surface is also very rapid. We have observed a surface turn over of CD14 in the range of 3 h in cycloheximide-treated cells. However, the internalization of CD14 from the plasma membrane was more rapid in cells treated with GA, suggesting that the effect of this drug is different from inhibition of CD14 import from the cell surface. Coincubation of J774 cells with GA and cycloheximide resulted in the disappearance of CD14 from the surface with the same kinetics as GA alone, suggesting that GA-mediated CD14 internalization does not require new protein synthesis. Because GA also blocks Hsp90, it is possible that the disappearance of surface CD14 is due to inhibition of this Hsp. Hsp90 has been observed to be associated to several membrane proteins (Citri *et al.*, 2002) and in close proximity to the cell surface (unpublished observations). Thus, Hsp90 may be involved in the transport and fusion of vesicles with the plasma membrane. Another possibility is that the effect of GA on surface CD14 is not due to Hsp90, but rather to Hsp70, which expression is increased in cells treated with this drug (see Figure 8C). Prior studies have also shown a similar increase in Hsp70 levels after treatment with GA (Sittler *et al.*, 2001). It is possible that the inhibition of both Hsp90 and Grp94 results in the increase of unfolded proteins within the cell, triggering the expression of Hsp70 (De Maio, 1999). The Hsp70 family, Hsp70 and Hsc70, has been observed in close proximity to the plasma membrane. Moreover, these proteins have been recently found to interact specifically with phospholipids within liposomes (Arispe *et al.*, 2002). Hsc70 has also been reported to form ion conductance channels in artificial lipid bilayers (Arispe and De Maio, 2000). Thus, a particular role for Hsp70 in the transport of vesicles containing CD14 cannot be discarded.

In summary, our observations indicate that treatment with GA resulted in the accumulation of CD14 within the ER. We speculate that the retention of CD14 in this compartment is due to lack of proper folding by inhibiting Grp94. The retention of CD14 within the ER reduces the transport of this glycoprotein to the plasma membrane. In addition, CD14 is rapidly internalized after GA treatment resulting in cells that cannot respond to LPS. The internalization of CD14 seems to be triggered by an additional effect of GA. It could be speculated that pharmacological treatment of individuals with GA results in a reduction of their innate immune response, at least the one mediated by CD14. Thus, treatment with GA may be beneficial during overwhelming infections by Gram-negative bacteria. On the other hand, blocking the immune response may be detrimental for the control of infection. Thus, the potential salutary or detrimental effect of GA in the treatment of sepsis remains to be established.

ACKNOWLEDGMENTS

We thank William B. Fulton for critically reading the manuscript. This work was supported by National Institutes of Health NIGMS grant GM-50878 and the Garrett Research Foundation.

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