Enhanced Macrophage Resistance to *Pseudomonas* Exotoxin A Is Correlated with Decreased Expression of the Low-Density Lipoprotein Receptor-Related Protein

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Cellular intoxication by exotoxin A of *Pseudomonas aeruginosa* (PEA) begins when PEA binds to its cellular receptor, the low-density lipoprotein receptor-related protein (LRP). This receptor is particularly abundant on macrophages. We hypothesize here that inducible changes in cellular expression levels of the LRP represent an important mechanism by which macrophage susceptibility to PEA is regulated by the host. We have examined the effect of lipopolysaccharide (LPS) on LRP expression and PEA sensitivity in the macrophage-like cell line HS-P. Using a [3H]leucine incorporation assay to measure inhibition of protein synthesis, we have demonstrated that HS-P LRP-specific ligand internalization studies. These data demonstrate for the first time that modulation of LRP levels by extracellular signaling molecules can alter cellular PEA sensitivity.

*Pseudomonas* exotoxin A (PEA) is an extracellular virulence factor produced by the opportunistic pathogen *Pseudomonas aeruginosa*. PEA irreversibly inhibits eukaryotic protein synthesis by ADP-ribosylating cytosolic elongation factor 2, leading to cell death (14). PEA is secreted as a 66-kDa proenzyme, which is extensively modified by target cells in order to generate and deliver the activated 37-kDa enzymatic fragment to the cytosol (28). The initial step in the intoxication process involves PEA binding to specific cell surface receptors followed by receptor-mediated endocytosis (23). A cell surface PEA binding protein was isolated from mouse fibroblasts (41) and liver cells (8) and subsequently identified as the low-density lipoprotein (LDL) receptor-related protein (LRP) (17). The isolation of LRP-deficient cells that are highly resistant to PEA confirmed the role of LRP as a cellular PEA receptor that mediates cytotoxicity (7, 45). Recently, Avramoglu et al. have restored PEA sensitivity in an LRP-deficient Chinese hamster ovary cell line by expressing functional chicken LRP (1). The LRP is a large cell surface glycoprotein belonging to the LDL receptor gene family. The LRP is synthesized as a 600-kDa proreceptor, which is posttranslationally processed into 515- and 85-kDa chains that remain associated through non-covalent interactions (9). The heavy chain is expressed entirely on the cell surface and is capable of binding an extraordinary range of structurally and functionally diverse ligands, including lipoproteins, lipases, proteinase inhibitors, proteinase inhibitor complexes, α2-macroglobulin (α2M) growth factor complexes, pathogens, and PEA (3, 10, 11, 16–18, 20, 27, 29, 35, 39). The 39-kDa receptor-associated protein (RAP) copurifies with the LRP and acts as an antagonist for all ligands binding to the LRP, including PEA (17). It has been proposed that the LRP may play a role in such diverse physiological processes as tissue remodeling, cellular growth regulation, and the metabolism of lipoproteins and proteinases.

An important determinant of cellular PEA susceptibility is the constitutive level of functional LRP expressed on the cell surface of different target cells. Mucci et al. discovered that a positive correlation exists between LRP expression and PEA sensitivity; cells constitutively expressing low levels of the LRP are highly resistant to PEA (26). Due in part to their different LRP expression levels (25), mammalian tissues and cells display a wide range of sensitivities to PEA (13, 24, 30, 33). In particular, the observation that the liver is the most common site of damage due to systemic PEA (13, 30, 33) is largely attributable to high levels of cellular LRP expression in hepatocytes and Kupffer cells (6, 25).

Various signaling molecules such as hormones (5, 21), growth factors (4, 44), and matrix components (34) have been shown to alter LRP levels in diverse cell types. Macrophage LRP expression is subject to regulation by specific cytokines and bacterial products. We previously reported that lipopolysaccharide (LPS) and interferon-gamma markedly decreased LRP expression at the mRNA, antigen, and functional levels in the RAW 264.7 macrophage-like cell line and in bone marrow macrophages (12, 19). We hypothesize here that inducible changes in cellular expression of LRP represent an important mechanism by which cellular susceptibility to PEA is regulated.
were then incubated with [3H]leucine for 21 h and then harvested as described with 50 ng of PEA per ml and various concentrations of RAP-GST for 1 h. Cells were treated either for 24 h at various concentrations of PEA or with 50 ng of PEA per ml for various periods of time. Following challenge, toxin was removed, and cells were detached by trypsin and passed every 2 to 3 days. This cell line was recently isolated from a spontaneous histiocytic sarcoma from the liver of a rat (47) and was chosen for this study based on its high sensitivity to PEA and its monocyte/macrophage origins. For experiments, cells were seeded at a concentration of 4 *10^5* cells/well into 96-well plates (for cytotoxicity assays) or at 2 *10^5* cells/well into 24-well plates (for ligand internalization studies) and were incubated overnight before treatment. The cell suspension was prepared by using the pGEX expression vector, (a kind gift from D. K. Strickland, American Red Cross, Rockville, Md.) from bacterial lysates with a GST purification module following the manufacturer’s instructions (Pharmacia Biotech, Baie d’Urfé, Quebec, Canada). All chemicals were obtained from the Sigma Chemical Co., St. Louis, Mo.

Cell culture. HS-P macrophage-like cells were cultured in T-75 flasks in RPMI medium, supplemented with 10% heat-inactivated fetal bovine serum, 50 U of penicillin per ml and 50 µg of streptomycin per ml at 5% CO2, 95% humidity, and 37°C. Media, serum, and supplements were all obtained from Gibco/BRL, Burlington, Ontario, Canada. Cells were detached with trypsin and passed every 2 to 3 days. This cell line was isolated from a spontaneous histiocytic sarcoma from the liver of a rat (47) and was chosen for this study based on its high sensitivity to PEA and its monocyte/macrophage origins. For experiments, cells were seeded at a concentration of 4 *10^5* cells/well into 96-well plates (for cytotoxicity assays) or at 2 *10^5* cells/well into 24-well plates (for ligand internalization studies) and were incubated overnight before treatment. The cell suspension was prepared by using the pGEX expression vector, (a kind gift from D. K. Strickland, American Red Cross, Rockville, Md.) from bacterial lysates with a GST purification module following the manufacturer’s instructions (Pharmacia Biotech, Baie d’Urfé, Quebec, Canada). All chemicals were obtained from the Sigma Chemical Co., St. Louis, Mo.

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RESULTS

HS-P PEA sensitivity. With a [3H]leucine incorporation assay to measure inhibition of protein synthesis, the cytotoxic effect of PEA on HS-P macrophage-like cells was determined. Results from experiments in which HS-P cells were treated with various concentrations of purified PEA for 2 h are displayed in Fig. 1A and indicate that HS-P cells are sensitive to PEA in a dose-dependent manner. To examine the effect duration of toxin exposure has on macrophage PEA cytotoxicity, HS-P cells were treated with 50 ng of PEA per ml for the indicated times (Fig. 1B). In both cases, HS-P cells are clearly sensitive to PEA, suggesting they possess the required cellular machinery for successful PEA intoxication, including functional cell surface LRPs and are a suitable macrophage cell line with which to evaluate factors which might alter PEA susceptibility.

PEA sensitivity is decreased by LPS. We next wished to determine if treatment of macrophages with LPS modifies their sensitivity to PEA. HS-P cells were pretreated for 24 h with LPS and then challenged with 100 ng of PEA per ml for 2 h. Increasing concentrations of LPS caused a decrease in HS-P PEA sensitivity (Fig. 2A). The duration of pretreatment also affected PEA sensitivity; cells exposed to LPS for an increased time acquired a greater resistance to PEA (Fig. 2B). These results indicate that LPS pretreatment decreases macrophage PEA sensitivity in a dose- and time-dependent fashion. To further examine this experiment, the ID50 values were determined for both HS-P cells pretreated with LPS at a concentration of 100 ng/ml for 24 h and for untreated cells (Fig. 2C). The ID50 value of HS-P cells pretreated with LPS was 25.7 ± 2.0 ng/ml, compared to a value of 11.3 ± 1.2 ng/ml for untreated cells. The ID50 value for pretreated cells is significantly different (P < 0.05) from that of control cells, indicating that LPS pretreatment increases HS-P PEA resistance twofold. In order to ensure that the extent of LRP down-regulation was not offset in this assay by increased receptor turnover, we assessed PEA toxicity after brief exposure to a saturating concentration of PEA (1,000 ng/ml) in control and LPS-treated cells. As demonstrated in Fig. 2D, the results indicate [3H]leucine incorporation is threefold higher in LPS-treated cells versus untreated cells. Taken together, these results demonstrate that exposure to LPS confers partial protection from PEA-mediated toxicity in macrophages and that the protection conferred is highest when the assay conditions are designed to reflect the number of cell surface receptors at a given time.

HS-P macrophages are protected from PEA by the LRP antagonist RAP. The RAP can act in vitro as a natural antagonist that prevents binding of all known ligands to the LRP. It has previously been reported that RAP prevents PEA binding...
Inhibited protein synthesis at all concentrations greater than 10 ng/ml and at all time points. 

Analysis of three independent experiments revealed that the level of cellular LRP mRNA decreased to 18.5% \( \pm 3.3\% \) of time zero values 6 h after LPS treatment (Fig. 4B), demonstrating for the first time that, in macrophages, LPS-dependent LRP modulation occurs very rapidly, at the mRNA level, after treatment. Previous results in our laboratory, including studies with the macrophage-like cell line RAW 246.7, indicate that LRP protein and functional levels decrease concomitantly with LRP mRNA (19). To verify that LRP down regulation occurs at the functional level in HS-P cells after LPS treatment, internalization studies were conducted with the LRP-specific ligand \( \alpha_2\)M*. The results from these experiments (Fig. 5) demonstrate that treatment with 100 ng of LPS per ml for 24 h reduces HS-P \( \alpha_2\)M* internalization three-and-a-half-fold compared to nontreated HS-P cells.

**DISCUSSION**

Macrophages constitute an important component of the host defense against bacterial pathogens such as *P. aeruginosa*. It is therefore not surprising that the macrophage is a target of *P. aeruginosa* virulence factors (37). Specifically, it has been demonstrated previously that PEA is cytotoxic to macrophages (31) and hampers their ability to carry out critical cellular processes. For example, PEA inhibits the ability of macrophages to engage in phagocytosis (31) and alters their secretion profiles of various cytokines, including interleukin-1 and tumor necrosis factor (38). Using an in vitro assay to measure the inhibition of protein synthesis, we report here that HS-P macrophage-like cells are sensitive to PEA in a time- and dose-dependent manner, similar to other cells of macrophage origin. However, macrophage cell lines appear to have marked differences in PEA sensitivity. With our assay, we have determined that the macrophage-like cell line RAW 246.7 is approximately 10-fold less sensitive to PEA (data not shown) than HS-P cells.

PEA intoxication is a complex multistep process that relies on the efficient participation of the target cell. Therefore, susceptibility to PEA should be based, at least in part, on the number of functional target cell components available for toxin entry. The observation that HS-P cells are highly sensitive to PEA, have abundant LRP mRNA levels, and are capable of internalizing the LRP-specific ligand \( \alpha_2\)M* suggests that these cells express high levels of the LRP. In addition, the ability of RAP-GST to block the cytotoxic effects of PEA confirms the LRP dependence of PEA toxicity in this cell type. Our initial hypothesis suggested that LPS exposure would act to protect macrophages from PEA through down-regulation of cell surface LRP. Northern blot analysis revealed that LRP mRNA levels dramatically and quickly decrease following LPS treatment. In addition, functional cell surface LRP levels decrease concomitantly with LRP mRNA as determined by \( \alpha_2\)M* internalization studies. These results extend our initial studies reporting that LPS treatment down-regulates the quantity of functional cell surface LRP in other macrophages (19).

LPS, a component of the outer membrane of gram-negative bacteria, is a well-recognized activating agent for macrophages, initiating a series of events which increase their ability to effectively combat invading pathogens. The primary macrophage LPS receptor is the glycosylphosphatidylinositol-anchored glycoprotein CD14 (46); however, activation can also occur via a CD14-independent pathway. The end result of LPS-induced signal transduction is an altered expression pattern for a variety of genes, including increased expression of proinflammatory cytokines and enzymes responsible for generating reactive oxygen and nitrogen species. While many of these changes in...
gene expression clearly enhance the ability of macrophages to destroy invading pathogens, the role of decreased cellular expression of some genes, particularly those for receptors (19, 36, 43), is far less clear. If, in fact, cell surface receptors constitute important portals of entry for pathogens or their products, then the potential advantage of actively decreasing the number of such sites is apparent. 

In the present study, we have identified one such potential mechanism. Pretreatment with LPS significantly decreased macrophage PEA sensitivity in a dose- and time-dependent manner. In addition, based on ID_{50} values, we observed that LPS pretreatment for 24 h at a concentration of 100 ng/ml decreased toxin sensitivity twofold. In order to further implicate receptor-dependent mechanisms in the observed differences in cellular toxin sensitivity, we also investigated cellular toxin susceptibility after a short duration of exposure to PEA. In this way, cellular receptors should be saturated with toxin and differences in receptor numbers may be more directly reflected by changes in cellular susceptibility than in studies utilizing longer periods of toxin exposure. Our results suggest a threefold higher susceptibility of untreated cells versus LPS-

FIG. 2. Altered HS-P PEA sensitivity following pretreatment with LPS. HS-P cells were treated for either 24 h with LPS at the indicated concentrations (A) or with 100 ng of LPS per ml for 6, 12, 18, or 24 h (B). Following LPS exposure, cells were challenged with 100 ng of PEA per ml for 2 h. (C) HS-P cells were pretreated with 100 ng of LPS per ml for 24 h (open squares) or were untreated (solid squares). Cells were then challenged with PEA for 2 h at the indicated concentrations. Following toxin exposure, cells were pulsed with media containing [3H]leucine for 21 h. (D) LPS-treated (100 ng/ml, 24 h) and nontreated cells were challenged with 1,000 ng of PEA per ml for 15 min, washed, and exposed to [3H]leucine for 12 h. Each data point represents the mean ± standard error of three separate experiments. *, significantly different from untreated cells (P < 0.05).

FIG. 3. Effect of RAP-GST on PEA-induced cytotoxicity in HS-P cells. Following overnight incubation, HS-P cells were treated with both PEA (50 ng/ml) and various concentrations of RAP-GST. Protein synthesis levels were determined by measuring the incorporation of [3H]leucine into cellular protein. Each data point represents the mean ± standard error of three separate experiments. Protein synthesis in the presence of RAP-GST was significantly (P < 0.05) higher at the 1,000-ng/ml concentration.
stimulated cells, further supporting our contention that receptor levels are positively correlated with toxin sensitivity. This observed decrease in toxin sensitivity correlates extremely well with the functional decrease in LRP-dependent ligand internalization in this cell type. We have also consistently observed that LPS exposure decreased RAW 264.7 PEA resistance; however, this effect was neither as reproducible nor as extensive as that reported here for HS-P cells. It is not yet clear why this is the case, but the relative resistance of RAW cells to PEA described above may play a role in masking any LPS-mediated protection. It should also be emphasized here that LPS- and cytokine-induced activation does not universally enhance cellular resistance to bacterial toxins; cellular sensitivity to Shiga and Shiga-like toxins in vascular endothelial cells is increased following LPS or cytokine treatment (22, 32, 40, 42).

Although it is not yet known whether LPS-mediated downregulation of LRP occurs in vivo, the protective effect of LPS reported here would have obvious beneficial effects on macrophage viability during PEA challenge. In such a scenario, macrophages, which have diminished levels of the LRP, would be relatively protected from PEA because they lack an efficient route for toxin internalization. The hypothesis that inducible cellular changes in LRP expression confer relative protection against PEA is also supported by our recent studies on hepatocytes, which demonstrated that matrix-dependent changes in LRP expression correlated with PEA resistance (18a, 34). Since extracellular signaling molecules have the ability to modulate LRP levels, it is plausible that this regulatory mechanism may be a factor in determining cellular and even tissue PEA sensitivity in vivo. Indeed, the results of the present study may suggest an additional mechanism by which LPS confers enhanced resistance to PEA challenge in vivo (48). It is probable that the production of various LRP regulatory factors may be initiated in response to P. aeruginosa, thus modulating PEA cellular sensitivity during infection. It is premature to predict whether such alterations in cellular PEA sensitivity would ultimately benefit the host or the pathogen. Since it is suspected that the LRP is also utilized for cellular entry by other pathogenic organisms, such as malaria (35) and minor group cold viruses (10), the importance of LRP regulation may not be restricted to PEA susceptibility.

Although the correlation between induced changes in LRP expression and PEA sensitivity is high, it should be emphasized that the LPS-induced decrease in macrophage PEA sensitivity seen here may be a product of many changes in macrophage function that can be mediated by LPS. Changes in any of the other steps involved in the PEA intoxication pathway might readily augment or oppose the protective effect resulting from decreased LRP expression. Nevertheless, it is clear from these studies, that changes in the expression of cellular receptors which act as portals of entry for pathogenic factors constitute a strong potential mechanism of host defense during P. aeruginosa infection.

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