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Structural Regions of MD-2 That Determine the Agonist-Antagonist Activity of Lipid IVa*

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A cell surface receptor complex consisting of CD14, Toll-like receptor (TLR4), and MD-2 recognizes lipid A, the active moiety of lipopolysaccharide (LPS). *Escherichia coli*-type lipid A, a typical lipid A molecule, potently activates both human and mouse macrophage cells, whereas the lipid A precursor, lipid IVa, activates mouse macrophages but is inactive and acts as an LPS antagonist in human macrophages. This animal species-specific activity of lipid IVa involves the species differences in MD-2 structure. We explored the structural region of MD-2 that determines the agonistic and antagonistic activities of lipid IVa to induce nuclear factor-κB activation. By expressing human/mouse chimeric MD-2 together with mouse CD14 and TLR4 in human embryonic kidney 293 cells, we found that amino acid regions 57–79 and 108–135 of MD-2 determine the species-specific activity of lipid IVa. We also showed that the replacement of Thr^{57}, Val^{61}, and Glu^{122} of mouse MD-2 with the corresponding human MD-2 sequence or alanines impaired the agonistic activity of lipid IVa, and antagonistic activity became evident. These mutations did not affect the activation of nuclear factor-κB, TLR4 oligomerization, and inducible phosphorylation of IκBα in response to *E. coli*-type lipid A. These results indicate that amino acid residues 57, 61, and 122 of mouse MD-2 are critical to determine the agonist-antagonist activity of lipid IVa and suggest that these amino acid residues may be involved in the discrimination of lipid A structure.

Bacterial lipopolysaccharide (LPS)[2] is a constituent of the outer membrane of the cell wall of Gram-negative bacteria and plays a major role in septic shock (1, 2). Engagement of LPS on the host cell results in rapid activation of a number of transcription factors, including NF-κB, which leads to production of inflammatory cytokines (3). Significant progress has been made in the identification of cell surface molecules that recognize LPS and transmit its signal to intracellular components. CD14, Toll-like receptor 4 (TLR4), and MD-2 participate in this molecular event and all of these molecules are necessary for cells to respond to picomolar concentrations of LPS (4, 5). A recent report (6) has suggested that sequential interactions of LPS with each of these molecules are required for optimal molecular recognition. LPS is first opsonized by the serum LPS-binding protein and then transferred to a CD14 molecule. This LPS-CD14 complex is further recognized by MD-2 to generate an LPS-MD-2 complex that produces TLR4-dependent cell stimulation. It has also been reported that MD-2 is necessary for TLR4 to undergo proper glycosylation and trafficking to the cell surface (7–9). Without MD-2, TLR4 is not able to reach the plasma membrane and resides predominantly in the Golgi apparatus. Thus, MD-2 is considered to play an important role for transferring LPS from CD14 to TLR4 and for correct cellular distribution of TLR4.

MD-2 also plays an important role for discriminating lipid A structure. The lipid A portion has been identified as the active center responsible for most LPS-induced biological effects (1, 10). *Escherichia coli*-type lipid A, a typical lipid A molecule, and its biosynthetic precursor lipid IVa have been synthesized chemically (compound 506 and 406, respectively), and their biological activities have been investigated extensively. Compound 506 and most varieties of LPS show little animal species-specific activity, whereas lipid IVa, as well as *Salmonella*-type lipid A, shows very little stimulatory activity and behaves as an antagonist in human macrophages, despite being potently active in murine macrophages (11, 12). This species-specific activity of lipid IVa and *Salmonella*-type lipid A has been attributed to the species difference in the structures of TLR4 (13, 14) and MD-2 (4, 15–17). Thus it is considered that MD-2 is also playing an important role for discriminating lipid A structure. To understand the molecular basis for this discriminating mechanism, we, in the present study, explored the structural region of MD-2 which determines the agonistic and antagonistic activities of lipid IVa.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—The HEK293 cell line (obtained from the Human Science Research Resources Bank, Tokyo, Japan) was grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin. Compound 506 and lipid IVa (compound 406) were obtained from Peptide Institute (Osaka, Japan). An antiserum against EIAV-tag epitope (amino acid sequence: ADRRIPGTAEE) was a kind gift from Dr. Nancy Rice (NCI-Frederick Cancer Research and Development Center). Stable transfectants expressing mouse CD14, EIAV-tagged mouse TLR4, FLAG-tagged mouse TLR4, and either EIAV-tagged mouse MD-2 or EIAV-tagged mouse MD-2-T57A,V61A,E122A were established as follows. After linearizing with PvuI, expression plasmids encoding the proteins described above were transfected into HEK293 cells by the calcium phosphate precipitation method. Stable transfectants were selected for G418 resistance at a concentration of 2 mg/ml. A monoclonal antibody (clone 5A5) that recognizes phosphorylated Ser^{32}-Ser^{36} of IκBα was purchased from Cell Signaling Technology (Danvers, MA).

Expression Plasmids—Expression plasmids encoding CD14, TLR4, and MD-2 as well as NF-κB-dependent luciferase reporter plasmid pELAM-L were described previously (16). Expression plasmids encoding MD-2 mutants were created by PCR-mediated mutagenesis, and mutations were confirmed by DNA sequencing.

NF-κB Reporter Assay—The NF-κB-dependent luciferase reporter assay was performed as described elsewhere (18). Briefly, HEK293 cells ($1–3 \times 10^5$/well) were plated in 12-well plates and on the following day

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[2] The abbreviations used are: LPS, lipopolysaccharide; HEK293, human embryonic kidney 293 cells; hMD-2, human MD-2; IκBα, inhibitor of NF-κB α; mMD-2, mouse MD-2; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; TLR, Toll-like receptor.

transfected by the calcium phosphate precipitation method with 10 ng each of CD14, TLR4, and MD-2 mutant expression plasmids together with 0.1 μg of pELAM-L and 2.5 ng of phRL-TK (Promega, Madison, WI) for normalization. At 24 h after transfection, cells were stimulated for 6 h, and the reporter gene activity was measured according to the manufacturer's (Promega) instructions.

Detection of MD-2 Proteins Expressed on the Cell Surface—Detection of cell surface MD-2 was performed as described previously (19) with a slight modification. Briefly, HEK293 cells were plated in 6-cm dishes and transfected with indicated plasmids by the calcium phosphate precipitation method. After 24 h, the cells were transferred to 1.5-ml tubes and then washed twice with PBS. After suspension with 0.5 ml of PBS containing Ca^{2+} and Mg^{2+}, cells were exposed to 0.5 mg/ml of a membrane-impermeable biotinylation reagent (sulfo-NHS-LC-LC-biotin; Pierce) at 4 °C for 15 min. The reaction was quenched by adding 1 ml of culture medium, and then cell extracts were prepared with 0.35 ml of PBS containing 1% Nonidet P-40, 2 mM EDTA, and a protease inhibitor mix (Roche Applied Science). After centrifugation at 12,000 × *g* for 5 min, the supernatants obtained were incubated with immobilized streptavidin-agarose at 4 °C for 1 h. The agarose was washed three times with PBS containing 1% Nonidet P-40, 2 mM EDTA, and subsequently biotinylated proteins were eluted from the agarose by incubating with 5 mg/ml of a water-soluble biotin derivative (sulfo-NHS-biotin; Pierce) dissolved in a buffer (50 mM Tris, pH 8, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40). The supernatant obtained was subjected to SDS-PAGE followed by Western blot analyzes.

Immunoprecipitation—HEK293 cells (2–5 × 10^7 cells) stably expressing mouse CD14, EIAV-tagged mouse TLR4, FLAG-tagged mouse TLR4, and either EIAV-tagged mouse MD-2 or EIAV-tagged mouse MD-2-T57A,V61A,E122A were suspended into 1 ml of culture medium. After stimulation with compound 506 or lipid IVa, cells were washed with cold PBS, and cell extracts were prepared with PBS containing 0.5% Nonidet P-40, 1 μM okadaic acid, and a protease inhibitor mix (Roche Applied Science). To the cell extracts, anti-FLAG M2-agarose (Sigma) was added, and the mixture was incubated at 4 °C for 1 h. The agarose was washed three times with PBS containing 0.5% Nonidet P-40, and subsequently bound proteins were eluted from the agarose by incubating with an elution buffer (0.1 M glycine, pH 3.5, 0.5% Nonidet P-40). The supernatant obtained was subjected to SDS-PAGE followed by Western blot analyses.

RESULTS

Responsiveness to Lipid A Molecules in HEK293 Cells Expressing CD14, TLR4, and MD-2—We first attempted to confirm the involvement of MD-2 in the animal species-specific activity of lipid IVa in HEK293 cells, which only respond to lipid A for the activation of NF-κB when CD14, TLR4, and MD-2 molecules are present. In HEK293 cells transiently expressing mouse CD14, TLR4, and MD-2, both compound 506 and lipid IVa comparably stimulated the NF-κB-dependent reporter activity (Fig. 1*A*). When mouse MD-2 was replaced with human MD-2, compound 506 still actively stimulated cells, whereas the response to lipid IVa was substantially impaired (Fig. 1*B*).

To examine the antagonistic activity of lipid IVa, HEK293 cells expressing mouse CD14, TLR4, and either mouse MD-2 or human MD-2 were stimulated with compound 506 in the presence of increasing concentrations of lipid IVa (Fig. 2). In cells expressing mouse MD-2, NF-κB-dependent reporter activity stimulated with 10 ng/ml compound 506 was almost unaffected by lipid IVa. In contrast, when mouse MD-2 was replaced with human MD-2, lipid IVa inhibited the compound 506-induced activation of NF-κB in a concentration-dependent manner. These results indicate that the difference in MD-2 structure between human and mouse is involved in determining the agonist-antagonist activity of lipid IVa.

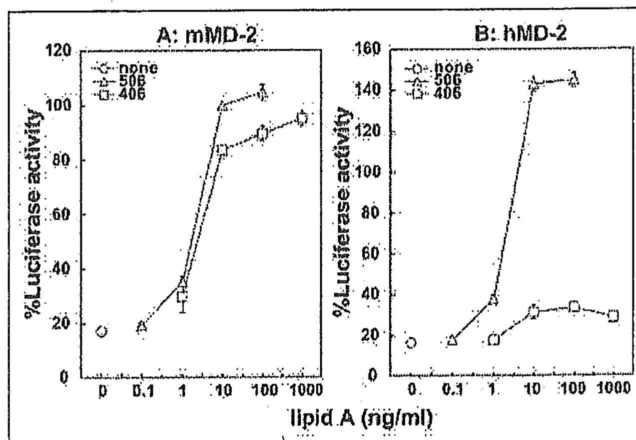

FIGURE 1. **Agonistic activities of lipid A and lipid IVa.** HEK293 cells were transiently transfected with mouse CD14, mouse TLR4, and either mouse MD-2 (*A*) or human MD-2 (*B*) expression plasmids together with an NF-κB-dependent luciferase reporter plasmid. After 24 h, cells were either unstimulated (○) or stimulated for 6 h with indicated concentrations of compound 506 (△) or lipid IVa (□), and luciferase activity was measured. The activity obtained with 10 ng/ml compound 506 in cells expressing mouse CD14, mouse TLR4, and mouse MD-2 was defined as 100%. Values are the means ± S.E. from seven independent experiments.

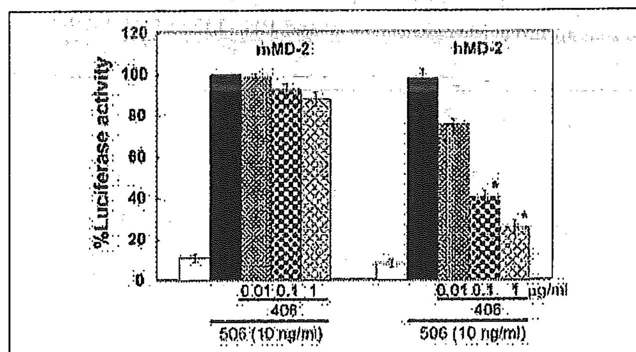

FIGURE 2. **Antagonistic activity of lipid IVa on lipid A-induced activation of NF-κB.** HEK293 cells were transiently transfected with mouse CD14, mouse TLR4, and either mouse MD-2 (*left five columns*) or human MD-2 (*right five columns*) expression plasmids together with an NF-κB-dependent luciferase reporter plasmid. After 24 h, cells were either unstimulated (*open columns*) or stimulated for 6 h with 10 ng/ml compound 506 (*506*) in the absence or presence of indicated concentrations of lipid IVa (*406*), and luciferase activity was measured. The activity obtained with 10 ng/ml compound 506 in cells expressing mouse CD14, mouse TLR4, and mouse MD-2 was defined as 100%. Values are the means ± S.E. from four independent experiments. * $p < 0.01$ (compared with the respective response in the absence of lipid IVa by two-tailed Student's *t* test).

MD-2 Structural Region Involved in Determining Agonist-Antagonist Activity of Lipid IVa—To explore the MD-2 structure required for the agonist-antagonist activity of lipid IVa, the coding region of mouse MD-2 was divided into six regions, and a series of MD-2 mutant plasmids in which each region was replaced with corresponding human MD-2 sequence was created (Fig. 3*A*). These chimeric mutants were expressed in HEK293 cells together with mouse CD14 and TLR4, and the NF-κB-dependent reporter activity was investigated (Fig. 3*B*). The cell surface expression of each of these MD-2 mutants was confirmed by Western blotting of biotinylated cell surface proteins, indicating that each of these mutants was similar enough to the parental mouse protein to be delivered to the cell membrane (Fig. 3*C*). Cells expressing each of the MD-2 mutants responded to compound 506 comparably with slight variations, indicating that all of these mutants functioned properly. In

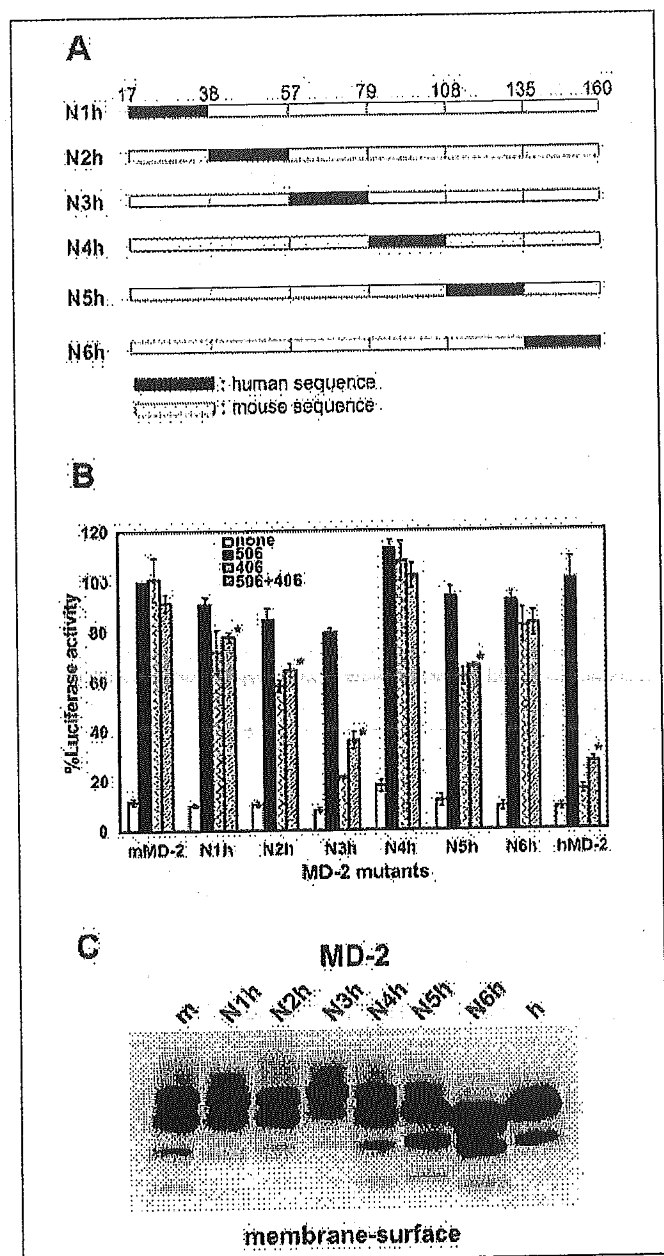

FIGURE 3. **Agonistic and antagonistic activities of lipid IVa in human/mouse chimeric MD-2.** *A*, schematic representation of human/mouse MD-2 constructs. The amino acid sequence of mouse MD-2 was divided into six regions at the indicated amino acid numbers, and each region was replaced with the corresponding human MD-2 sequence. The predicted signal peptide sequence (amino acids 1–16) was omitted. *B*, HEK293 cells were transiently transfected with mouse CD14, mouse TLR4, and the indicated mutant MD-2 expression plasmids together with an NF-κB-dependent luciferase reporter plasmid. After 24 h, cells were either unstimulated (*open columns*) or stimulated for 6 h with 10 ng/ml compound 506 (*506*), 1 μg/ml lipid IVa (*406*), or 10 ng/ml compound 506 in the presence of 1 μg/ml lipid IVa (*506 + 406*), and luciferase activity was measured. The activity obtained with 10 ng/ml compound 506 in cells expressing mouse CD14, mouse TLR4, and mouse MD-2 was defined as 100%. Values are the means ± S.E. from at least four independent experiments. * $p < 0.01$ (compared with the respective response in the absence of lipid IVa by two-tailed Student's *t* test). *C*, HEK293 cells were transiently transfected with mouse CD14, mouse TLR4, and the indicated mutant MD-2 expression plasmids. After 24 h, cell surface proteins were biotinylated with a membrane-impermeable biotinylation reagent, and biotinylated proteins from cell extracts were collected with streptavidin-agarose. After washing, biotinylated proteins were eluted from the agarose by incubating with a water-soluble biotin derivative, and the supernatant obtained was subjected to SDS-PAGE followed by Western blot analysis to detect membrane surface MD-2 mutant proteins. Similar results were obtained in two additional experiments.

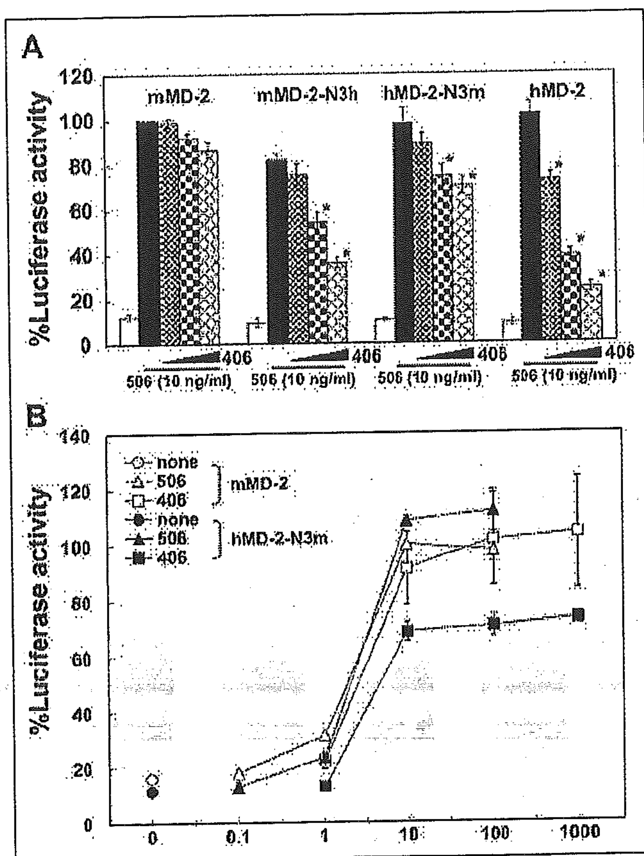

FIGURE 4. **N3 region of MD-2 is partly involved in animal species-specific activity of lipid IVa.** HEK293 cells were transiently transfected with mouse CD14, mouse TLR4, and the indicated mutant MD-2 expression plasmids together with an NF-κB-dependent luciferase reporter plasmid. After 24 h, cells were either unstimulated (*open columns*) or stimulated for 6 h with 10 ng/ml compound 506 (*506*) in the absence or presence of increasing concentrations (0.01, 0.1, and 1 μg/ml) of lipid IVa (*406*) in *A*, or were either unstimulated (○, ●) or stimulated for 6 h with the indicated concentrations of compound 506 (△, ▲) or lipid IVa (□, ■) in *B*, and luciferase activity was measured. The activity obtained with 10 ng/ml compound 506 in cells expressing mouse CD14, mouse TLR4, and mouse MD-2 was defined as 100%, and luciferase activity was measured. The activity obtained with 10 ng/ml compound 506 in cells expressing mouse CD14, mouse TLR4, and mouse MD-2 was defined as 100%. Values are the means ± S.E. from at least three independent experiments. * $p < 0.01$ (compared with the respective response in the absence of lipid IVa by two-tailed Student's *t* test).

contrast, the activity of lipid IVa varied and was substantially impaired in cells expressing the mMD-2-N3h mutant. The activity was similar to that observed in cells expressing human MD-2. A partial reduction with a statistical significance in the activity of lipid IVa was also observed in mMD-2-N2h and mMD-2-N5h mutants as well as in mMD-2-N1h to a lesser extent. The antagonistic activity of lipid IVa was also studied in these MD-2 mutants by stimulating with compound 506 in the presence of lipid IVa. (Fig. 3*B*). In cells expressing mouse MD-2, lipid IVa did not inhibit the compound 506-induced activation of NF-κB, whereas in cells expressing human MD-2 the activity of compound 506 was inhibited substantially by lipid IVa as mentioned above. When MD-2 mutants were expressed, the activity of compound 506 was inhibited by lipid IVa in cells expressing the mMD-2-N3h mutant to a degree similar to that observed with human MD-2. These results suggest that the N3 region of MD-2 is involved in the animal species-specific activity of lipid IVa.

We next asked whether the N3 region of MD-2 is critical for establishing the agonist-antagonist activity of lipid IVa. To address this, HEK293 cells expressing mouse CD14, TLR4, and N3 chimeras or

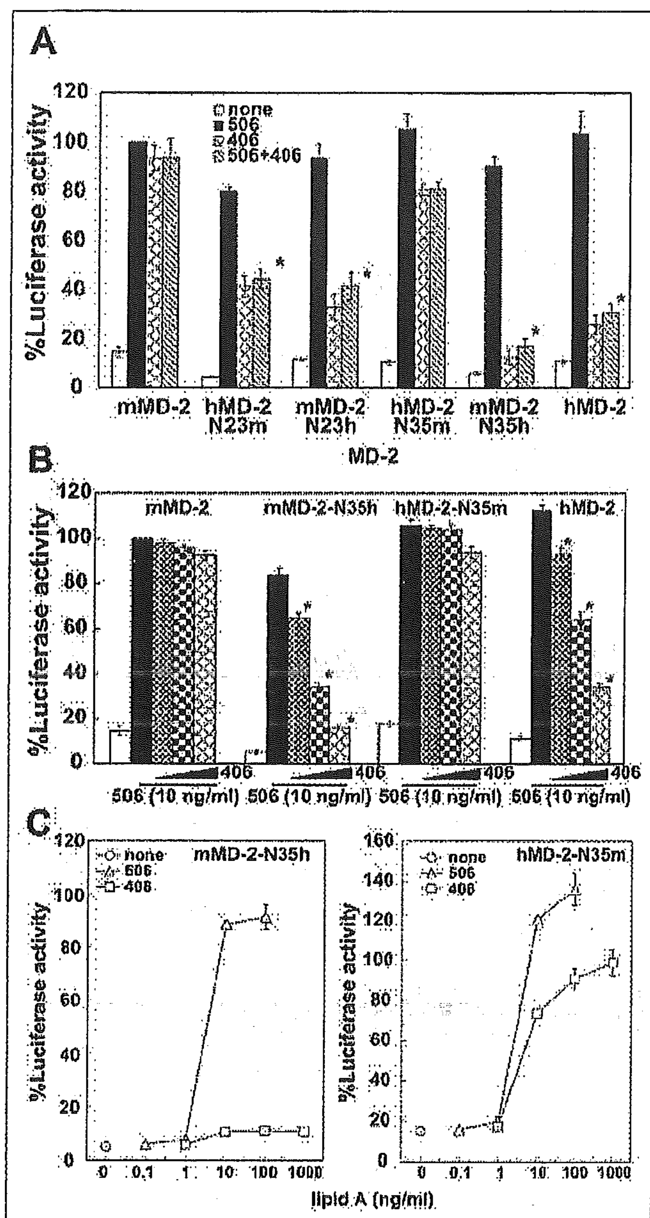

FIGURE 5. **N3 and N5 regions of MD-2 are responsible for animal species-specific activity of lipid IVa.** HEK293 cells were transiently transfected with mouse CD14, mouse TLR4, and the indicated mutant MD-2 expression plasmids together with an NF-κB-dependent luciferase reporter plasmid. After 24 h, cells were either unstimulated (*open columns*) or stimulated for 6 h with 10 ng/ml compound 506 (*506*), 1 μg/ml lipid IVa (*406*), or 10 ng/ml compound 506 in the presence of 1 μg/ml of lipid IVa (*506 + 406*) in *A*, or were either unstimulated (*open columns*) or stimulated for 6 h with 10 ng/ml compound 506 in the absence or presence of increasing concentrations (0.01, 0.1, and 1 μg/ml) of lipid IVa in *B*, or were either unstimulated (○) or stimulated for 6 h with the indicated concentrations of compound 506 (△) or lipid IVa (□) in *C*, and luciferase activity was measured. The activity obtained with 10 ng/ml compound 506 in cells expressing mouse CD14, mouse TLR4, and mouse MD-2 was defined as 100%. Values are the means ± S.E. from at least three independent experiments. * $p < 0.01$ (compared with the respective response in the absence of lipid IVa by two-tailed Student's *t* test).

parental MD-2 were stimulated with compound 506 in the presence of increasing concentrations of lipid IVa (Fig. 4*A*). As expected, lipid IVa concentration-dependently inhibited the compound 506-induced activation of NF-κB in cells expressing the N3h mutant; however, the inhibitory activity was relatively weaker than that observed in cells expressing the parental hMD-2. If the N3 region of MD-2 is the only region responsible for the species-specific activity of lipid IVa, it was expected that replacing the N3 region of human MD-2 with the corresponding mouse MD-2 sequence would show the mouse phenotype. However, a slight inhibitory effect of lipid IVa was still observed in cells expressing the N3m chimera (hMD-2-N3m). In addition, the agonistic activity of lipid IVa in cells expressing this N3m chimera only reached ~73% of the activity observed in cells expressing the parental mouse MD-2 (Fig. 4*B*).

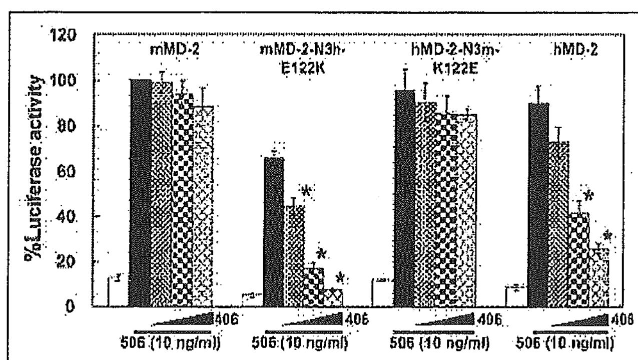

FIGURE 6. **Amino acid 122 of MD-2 is involved in animal species-specific activity of lipid IVa.** HEK293 cells were transiently transfected with mouse CD14, mouse TLR4, and the indicated mutant MD-2 expression plasmids together with an NF-κB-dependent luciferase reporter plasmid. After 24 h, cells were either unstimulated (*open columns*) or stimulated for 6 h with 10 ng/ml compound 506 (*506*) in the absence or presence of increasing concentrations (0.01, 0.1, and 1 μg/ml) of lipid IVa (*406*), and luciferase activity was measured. The activity obtained with 10 ng/ml compound 506 in cells expressing mouse CD14, mouse TLR4, and mouse MD-2 was defined as 100%. Values are the means ± S.E. from at least three independent experiments. * $p < 0.01$ (compared with the respective response in the absence of lipid IVa by two-tailed Student's *t* test).

The above result brought us to explore another MD-2 region, in addition to the N3 region, that is involved in the agonist-antagonist activity of lipid IVa. Because a slight antagonistic activity of lipid IVa was observed in mMD-2-N2h and mMD-2-N5h mutants (Fig. 3*B*), we created MD-2 mutant plasmids in which both the N2 and N3 regions or the N3 and N5 regions were mutated. These MD-2 mutants were used to examine the NF-κB-dependent reporter activity in HEK293 cells expressing mouse CD14, TLR4 (Fig. 5*A*). Compound 506 showed activity comparable with all of these MD-2 mutants. With the MD-2 mutant in which the N2 and N3 regions of human MD-2 were replaced with corresponding mouse sequences (hMD-2-N23m) and the mutant in which the N2 and N3 regions of mouse MD-2 were replaced with corresponding human sequences (mMD-2-N23h), lipid IVa showed partial agonistic and partial antagonistic activities. Contrarily, lipid IVa showed a strong agonistic activity with the MD-2 mutant in which the N3 and N5 regions of human MD-2 were replaced with corresponding mouse sequences (hMD-2-N35m), and almost no agonistic activity of lipid IVa, even at 1 μg/ml, was observed with a mutant in which the N3 and N5 regions of mouse MD-2 were replaced with corresponding human sequences (mMD-2-N35h). The antagonistic activity of lipid IVa was also examined with these mutants (Fig. 5*B*). Almost no antagonistic activity was observed with hMD-2-N35m, and a clear antagonistic activity was observed with mMD-2-N35h. In addition, lipid IVa caused almost no agonistic activity in cells expressing mMD-2-N35h and showed a potent agonistic activity comparable with that observed with wild-type mouse MD-2 (see Fig. 1*A*) in cells expressing hMD-2-N35m (Fig. 5*C*). These results indicate that both of the N3 and N5 regions of MD-2 are involved in determining the agonist-antagonist activity of lipid IVa.

MD-2 Structural Region Involved in Antagonistic Activity of Lipid IVa—Replacement of the N3 and N5 regions of mouse MD-2 with corresponding human sequences changed the activity of lipid IVa from agonistic to antagonistic without affecting the activity of compound

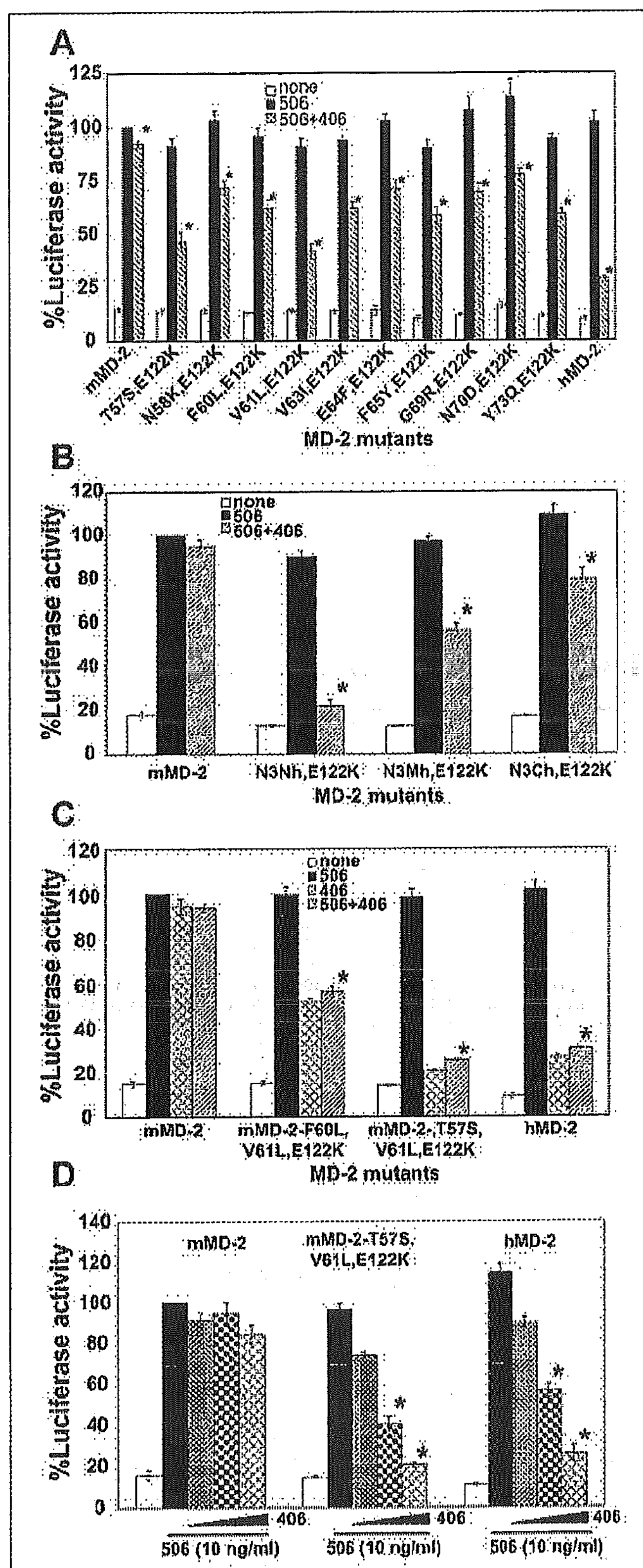

FIGURE 7. **Amino acid residues 57, 61, and 122 of MD-2 are involved in animal species-specific activity of lipid IVa.** HEK293 cells were transiently transfected with mouse CD14, mouse TLR4, and the indicated mutant MD-2 expression plasmids together with an NF-κB-dependent luciferase reporter plasmid. After 24 h, cells were either unstimulated (*open columns*) or stimulated for 6 h with 10 ng/ml compound 506 (*506*) or 10 ng/ml compound 506 in the presence of lipid IVa (*506 + 406*) in *A* and *B*, were either unstimulated (*open columns*) or stimulated for 6 h with 10 ng/ml compound 506, 1 μg/ml lipid IVa, or 10 ng/ml compound 506 in the presence of 1 μg/ml lipid IVa in *C*, or were either unstimulated (*open columns*) or stimulated for 6 h with 10 ng/ml compound 506 in the absence or presence of increasing concentrations (0.01, 0.1, and 1 μg/ml) of lipid IVa in *D*, and luciferase activity was measured. The activity obtained with 10 ng/ml compound 506 in cells expressing mouse CD14, mouse TLR4, and mouse MD-2 was defined as 100%. Values are the means ± S.E. from at least three independent experiments. * $p < 0.01$ (compared with the respective response in the absence of lipid IVa by two-tailed Student's *t* test).

506. Human and mouse MD-2 possess a similar amino acid sequence in their N5 regions with only a major difference at amino acid 122, a change in charge. Thus, to investigate the involvement of amino acid 122 of MD-2 in the activity of lipid IVa, we examined the antagonistic activity of lipid IVa with a mouse MD-2 mutant (mMD-2-N3h-E122K) in which the N3 region and amino acid 122 were replaced with the corresponding human sequence and a human MD-2 mutant (hMD-2-N3m-K122E) in which the N3 region and amino acid 122 were replaced with the corresponding mouse sequence (Fig. 6). A stronger antagonistic activity was observed in cells expressing mMD-2-N3h-E122K compared with those expressing mMD-2-N3h (see Fig. 4). On the other hand, almost no antagonistic effect was observed with hMD-2-N3m-K122E. It is therefore likely that the involvement of the N5 region is explained by amino acid 122.

We next asked whether the involvement of the N3 region was also explained at the amino acid level. To address this, each amino acid of the N3 region of mouse MD-2, carrying E122K mutation, was replaced individually with the corresponding human amino acid residue, and the antagonistic activity of lipid IVa was examined (Fig. 7*A*). Although compound 506-induced activation of NF-κB was inhibited to some extent in cells expressing these MD-2 mutants, sufficient antagonistic activities were not observed. Thus we created mouse MD-2 mutant plasmids in which the overlapping three regions (amino acid residues 57–65, 64–73, and 69–78) within N3 and amino acid 122 were replaced with the corresponding human sequences (each named as N3Nh,E122K, N3Mh,E122K, N3Ch,E122K), and the antagonistic activity of lipid IVa was examined (Fig. 7*B*). A potent antagonistic effect of lipid IVa was observed with the N3Nh,E122K mutant, indicating that amino acid residues 57–65 and 122 of human MD-2 play a role in the antagonistic effect. Because the N3 region of human MD-2 is leucine-rich, we suspected that two leucines (amino acids 60 and 61) might be involved in the antagonistic effect. Thus we created a mouse MD-2 mutant plasmid carrying F60L, V61L, and E122K mutations. Furthermore, because relatively potent antagonistic effects were observed with T57S,E122K and V61L,E122 mutants (Fig. 7*A*), we also created a mouse MD-2 mutant plasmid carrying T57S, V61L, and E122K mutations. Agonistic effects of compound 506 and lipid IVa as well as antagonistic effects of lipid IVa were examined (Fig. 7*C*). Only partial agonistic and antagonistic activities of lipid IVa were observed with the mMD-2-F60L,V61L,E122K mutant. However, these activities and the concentration-inhibition effect of lipid IVa (Fig. 7*D*) in cells expressing the mMD-2-T57S,V61L,E122K mutant were comparable with those observed in hMD-2, indicating a critical role of these three amino acid residues (Ser^{57}, Leu^{61}, and Lys^{122}) for expressing the antagonistic activity.

MD-2 Structural Region Involved in Agonistic Activity of Lipid IVa—Mutation of Thr^{57}, Val^{61}, and Glu^{122} of mouse MD-2 into corresponding human MD-2 sequences caused not only the appearance of antagonistic activity of lipid IVa but also the disappearance of its agonistic activity, without losing the agonistic activity of compound 506 (Fig. 7*C*). Thus we next asked whether these three amino acid residues

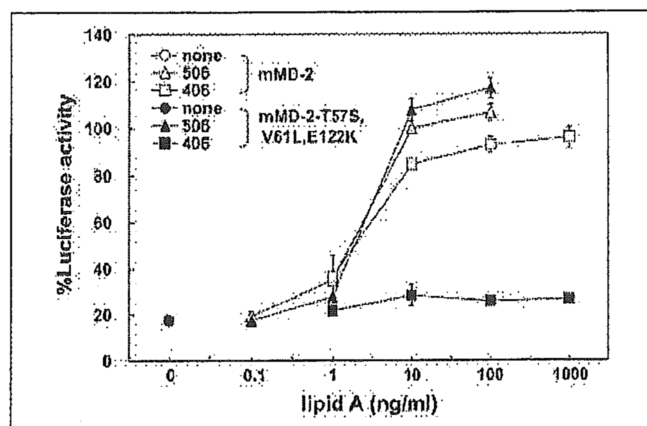

FIGURE 8. **Replacement of Thr[57], Val[61], and Glu[122] of mouse MD-2 with corresponding human MD-2 sequence loses the agonistic activity of lipid IVa without affecting lipid A activity.** HEK293 cells were transiently transfected with mouse CD14, mouse TLR4, and the indicated MD-2 expression plasmids together with an NF-κB-dependent luciferase reporter plasmid. After 24 h, cells were either unstimulated (○, ●) or stimulated for 6 h with indicated concentrations of compound 506 (△, ▲) or lipid IVa (□, ■), and luciferase activity was measured. The activity obtained with 10 ng/ml compound 506 in cells expressing mouse CD14, mouse TLR4, and mouse MD-2 was defined as 100%. Values are the means ± S.E. from four independent experiments.

of mouse MD-2 were selectively involved in the agonistic activity of lipid IVa. To address this, we examined the agonistic activities of lipid IVa and compound 506 in cells expressing mMD-2-T57S,V61L,E122K together with mouse CD14 and TLR4 (Fig. 8). In these cells, compound 506 induced potent activation of NF-κB comparable with that observed in cells expressing wild-type mouse MD-2, whereas almost no agonistic activity was observed with lipid IVa at concentrations from 1 to 1,000 ng/ml. Although the mutation of glutamic acid to a lysine caused a charge reversal, mutations from threonine to serine and from valine to leucine may not cause significant changes. It is, therefore, still possible that compound 506 may require these amino acid residues for its agonistic activity, but these changes in amino acid residues may be tolerated. To address this, we mutated these three amino acid residues in mouse MD-2 into alanines either individually or in combinations and examined the agonistic activities of compound 506 and lipid IVa as well as the antagonistic activity of lipid IVa (Fig. 9). Although the agonistic activity of compound 506 with the E122A mutation was slightly enhanced, none of the mutations caused significant changes in the activity of compound 506. No significant changes in the agonistic and antagonistic activities of lipid IVa were observed with each point mutant or the T57S,V61A mutant, whereas the concurrent mutation of all three amino acid residues substantially decreased the agonistic activity, and the antagonistic activity was also evident (Fig. 9*A*). The concentration-response effects showed that the activity of compound 506 was decreased only slightly by the concurrent mutation of all three amino acid residues, whereas the activity of lipid IVa was substantially impaired (Fig. 9*B*). These results indicate that these three amino acid residues are selectively involved in the agonistic activity of lipid IVa and critical for determining its agonist-antagonist activity.

Role of Thr[57], Val[61], and Glu[122] of MD-2 in TLR4 Signaling—The role of Thr[57], Val[61], and Glu[122] of mouse MD-2 in TLR4 signaling was studied in HEK293 cells stably expressing mouse CD14, EIAV-tagged mouse TLR4, FLAG-tagged mouse TLR4, and either EIAV-tagged mouse MD-2 or EIAV-tagged mouse MD-2-T57A,V61A,E122A. These cells were stimulated with compound 506 or lipid IVa, and TLR4 oligomerization was examined (Fig. 10). For this, FLAG-tagged TLR4 was immunoprecipitated, and coprecipitation of EIAV-tagged TLR4 was detected by Western blotting. Coprecipitations of EIAV-tagged TLR4 were

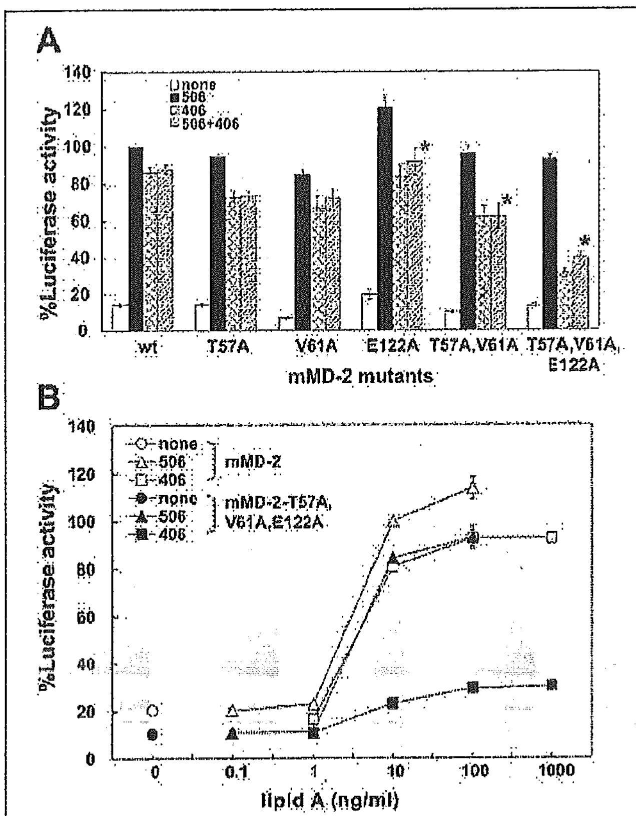

FIGURE 9. **Replacement of Thr[57], Val[61], and Glu[122] of mouse MD-2 with alanine loses the agonistic activity of lipid IVa without affecting lipid A activity.** HEK293 cells were transiently transfected with mouse CD14, mouse TLR4, and the indicated MD-2 expression plasmids together with an NF-κB-dependent luciferase reporter plasmid. After 24 h, cells were either unstimulated (*open columns*) or stimulated for 6 h with 10 ng/ml compound 506 (*506*), 1 μg/ml lipid IVa (*406*), or 10 ng/ml compound 506 in the presence of 1 μg/ml lipid IVa (*506 + 406*) in *A*, or were either unstimulated (○, ●) or stimulated for 6 h with the indicated concentrations of compound 506 (△, ▲) or lipid IVa (□, ■) in *B*, and luciferase activity was measured. The activity obtained with 10 ng/ml compound 506 in cells expressing mouse CD14, mouse TLR4, and mouse MD-2 was defined as 100%. Values are the means ± S.E. from three independent experiments. * $p < 0.01$ (compared with the respective response in the absence of lipid IVa by two-tailed Student's *t* test). *wt*, wild-type.

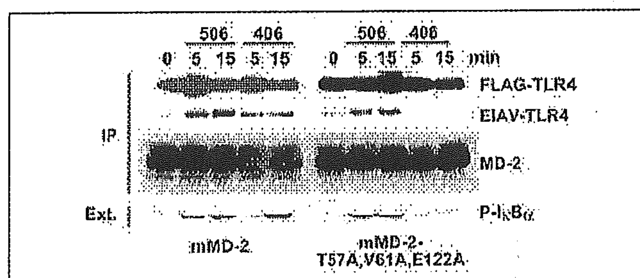

FIGURE 10. **Role of Thr[57], Val[61], and Glu[122] of mouse MD-2 in TLR4 signaling.** HEK293 cells stably expressing mouse CD14, EIAV-tagged mouse TLR4, FLAG-tagged mouse TLR4, and either EIAV-tagged mouse MD-2 (*left five lanes*) or EIAV-tagged mouse MD-2-T57A,V61A,E122A (*right five lanes*) were stimulated with 100 ng/ml compound 506 or 1 μg/ml lipid IVa for the indicated times. Then, cell extracts (*Ext.*) were prepared, and FLAG-tagged TLR4 was immunoprecipitated (*IP*). Precipitated FLAG-tagged TLR4 and coprecipitated EIAV-tagged TLR4 as well as MD-2 proteins were detected by Western blotting. A part of cell extracts prepared above were subjected to the detection of IκBα protein phosphorylated at Ser[32]-Ser[36] (*P-IκBα*) by Western blotting. Similar results were obtained in two additional experiments.

barely detectable without stimulations but were detectable after compound 506 stimulation in both stable transfectants. After lipid IVa stimulation, the coprecipitation was also detected in cells expressing wild-

type MD-2 but was barely detectable in cells expressing mMD-2-T57A,V61A,E122A. Both the wild-type and mutant MD-2 were coprecipitated with TLR4 without ligand stimulation, and the amount coprecipitated was unaffected by stimulations. In parallel with TLR4 oligomerization, the inducible phosphorylation of IκBα was observed in response to compound 506 in both stable transfectants. The phosphorylation was also observed in response to lipid IVa in cells expressing wild-type MD-2 but was barely detectable in cells expressing mMD-2-T57A,V61A,E122A. These results support the above conclusion that Thr^{57}, Val^{61}, and Glu^{122} of mouse MD-2 are selectively involved in the agonistic activity of lipid IVa and critical for determining its agonist-antagonist activity.

DISCUSSION

In the present study, we investigated the structural region of MD-2 required for agonistic and antagonistic activities of lipid IVa by utilizing its animal species-specific activity. The involvement of MD-2 in animal species-specific activity of lipid IVa has been demonstrated previously by expressing human and mouse MD-2 in human monocytic THP-1 cells (4), mouse pro B Ba/F3 cells (15), and HEK293 cells (17). In the present study, we confirmed that the lipid IVa-induced activation of NF-κB in HEK293 cells expressing mouse CD14, TLR4, and MD-2 was substantially impaired when mouse MD-2 was replaced with human MD-2. The activity of compound 506, a typical lipid A molecule, was not significantly affected by the replacement, indicating that both human and mouse MD-2 are functional on mouse TLR4. Thus, in the present study, we created mouse/human chimeric MD-2 mutant plasmids and found that both the N3 (amino acids 57–79) and N5 (amino acids 108–135) regions of MD-2 were involved in the species-specific activity of lipid IVa. We further narrowed the region down and found that the concurrent replacement of Thr^{57}, Val^{61}, and Glu^{122} of mouse MD-2 with the corresponding human MD-2 amino acids substantially decreased the agonistic activity of lipid IVa without affecting the activity of compound 506. The replacement of each of these amino acid residues individually or as pairs was not enough to lose the activity, indicating that these three residues together contribute to the species-specific activity of lipid IVa. A tertiary structure model of human MD-2, reported by Gruber *et al.* (20), shows that amino acid residues 57, 61, and 122 of MD-2 are sterically located in close proximity. Thus the domain created by these three amino acid residues may be involved in determining the agonist-antagonist activity of lipid IVa.

The mutation of Thr^{57} to Ser, Val^{61} to Leu, and Glu^{122} to Lys of mouse MD-2 substantially decreased the agonistic activity of lipid IVa, whereas these replacements did not affect the activity of compound 506. Because the difference in amino acid structure between Thr and Ser or between Val and Leu is only one methyl or methylene moiety, there was still the possibility that these changes in amino acid residues may be tolerated even though compound 506 may require these amino acid residues for full agonistic activity. Thus we examined the activity of compound 506 in a mouse MD-2 mutant in which Thr^{57}, Val^{61}, and Glu^{122} were replaced with alanines, and we found that the activity was not affected by these substitutions, whereas the activity of lipid IVa was substantially impaired. It is therefore likely that these three amino acid residues are selectively involved in the agonistic activity of lipid IVa.

The replacement of amino acid residues 57, 61, and 122 of mouse MD-2 with corresponding human MD-2 amino acids substantially decreased the agonistic activity of lipid IVa. However, replacement of amino acid residues 57, 61, and 122 of human MD-2 with the corresponding mouse MD-2 amino acid residues restored the agonistic activity of lipid IVa only to ~50% of the activity observed in mouse MD-2 (data not shown). Replacement of the N3 region, and replacement of amino acid 122 in addition to the N3 region of human MD-2 with corresponding mouse MD-2 sequence restored the activity to ~73% (Fig. 4*B*) and 90% (data not shown), respectively. Therefore, these three amino acid residues are necessary for the agonistic activity of lipid IVa, but additional amino acid residues in the N3 region may be required for its full agonistic activity.

It has been reported, in studies using soluble MD-2 (6, 21–23) and a peptide fragment of MD-2 (24) that LPS directly binds to MD-2 in a highly basic region (amino acids 119–132). In our study, the mutation of Thr^{57}, Val^{61}, and Glu^{122} of mouse MD-2 to alanines (Fig. 9) or the mutation of Ser^{57}, Leu^{61}, and Lys^{122} of human MD-2 to corresponding mouse MD-2 amino acid residues (data not shown) did not affect the agonistic activity of compound 506, indicating that these three amino acid residues are not involved in lipid A binding. In addition, it is unlikely that these three amino acid residues are involved in lipid IVa binding because lipid IVa showed an antagonistic effect in cells expressing the mouse MD-2 mutant in which all three of these amino acid residues were replaced with the corresponding human MD-2 amino acid residues or with alanines. For TLR4 signaling, the interaction between MD-2 and TLR4 (7, 22, 23, 25), as well as dimerization of TLR4 (26, 27) were reported to be important. For the interaction with TLR4, Cys^{95}, Tyr^{102}, and Cys^{105} of human MD-2 have been reported to be involved (22–23, 25). Miyake (5) and Gangloff and Gay (28) have proposed that MD-2 plays an important role in regulating TLR4 dimerization upon LPS binding. Because the ability of MD-2 to associate with TLR4 and compound 506-induced TLR4 dimerization as well as inducible phosphorylation of IκBα were not affected by the mutation of Thr^{57}, Val^{61}, and Glu^{122} of mouse MD-2 (Fig. 10), these amino acid residues are unlikely to be involved in interactions with TLR4 or in TLR4 dimerization. These amino acid residues may participate in the discrimination of lipid A structure.

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Alachlor and carbaryl suppress lipopolysaccharide-induced iNOS expression by differentially inhibiting NF-κB activation

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Abstract

Nitric oxide (NO) produced by macrophages plays an important role in host defense and inflammation. We found that two agrochemicals, alachlor and carbaryl, inhibit lipopolysaccharide (LPS)-induced NO production by macrophages. In the present study, we investigated this inhibitory mechanism in RAW 264 cells. Both chemicals inhibited LPS-induced iNOS protein and mRNA expression as well as murine iNOS promoter activity. When treating these chemicals with reducing agents, the inhibition by carbaryl was reversed, but not the inhibition by alachlor. These chemicals also inhibited LPS-induced interferon-β (IFN-β) expression, an indispensable factor for LPS-induced iNOS expression. The inhibited iNOS expression, however, was not restored by exogenous IFN-β supplementation. LPS-induced nuclear translocation of NF-κB, which is necessary for the expression of IFN-β and iNOS, was inhibited by these chemicals: however, the LPS-induced degradation of IκB-α and IκB-β was inhibited only by alachlor. These results indicate that alachlor and carbaryl differentially impair the LPS-induced NF-κB activation, leading to the inhibition of NO production.
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Keywords: Lipopolysaccharide; Inducible nitric oxide synthase; Macrophage; Alachlor; Carbaryl

Nitric oxide (NO) is a free radical gas and plays important roles for host defense, neurotransmission, and vasodilatation [1–3]. The neural and endothelial Ca^{2+}-dependent NO synthases are constitutively expressed in a variety of cell types. In contrast, inducible NO synthase (iNOS), which is a Ca^{2+}-independent enzyme, is expressed in response to specific stimuli in cells including macrophages [4,5]. Large amounts of NO catalyzed by iNOS in activated macrophages mediate bactericidal and tumoricidal activities [6]. It has been shown that iNOS expression is dramatically induced by bacterial lipopolysaccharide (LPS) and that this induction is mainly regulated at the transcriptional level [5,7,8]. Among transcriptional factors involved in the LPS-induced iNOS expression, NF-κB is considered to play a critical role. In fact, two NF-κB sites located in the promoter region of the murine iNOS gene are indispensable for maximal expression of iNOS induced by LPS [9,10]. In macrophages, LPS stimulation initiates the degradation of NF-κB inhibitor proteins such as IκB-α and IκB-β in the cytosol, leading to the translocation of the liberated NF-κB, consisting mostly of p50/p65 heterodimer [11], into the nucleus. Following these steps, NF-κB binds to the NF-κB motif located in the promoter of target genes to enhance their transcription. IFN-β is known to play an indispensable role in LPS-induced iNOS expression [5,12,13] by acting in autocrine/paracrine fashions. Activation of NF-κB by LPS is known to mediate transcription of the IFN-β gene [14–17]. Thus, the activation of NF-κB by LPS plays a critical role for the expression of both IFN-β and iNOS.

Alachlor and carbaryl are used as a herbicide and an insecticide, respectively, and are suspected of

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having endocrine disrupting effects [18,19]. Previously, we studied the effect of various possible endocrine disrupting chemicals on LPS-induced TNF-α and NO production by murine macrophages and found that alachlor and carbaryl strongly inhibit LPS-induced NO production in vitro [20]. Since little is known about how these agrochemicals affect macrophage functions, the present study investigates the mechanisms by which alachlor and carbaryl treatment inhibits LPS-induced NO production by a mouse macrophage cell line RAW 264.

Materials and methods

Cell culture and reagents. A mouse macrophage cell line RAW 264 (obtained from the Riken Cell Bank, Tsukuba, Japan) was cultured in DMEM (Gibco-BRL, Rockville, MD) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (Gibco-BRL), penicillin (100 U/ml), and streptomycin (100 μg/ml). Alachlor and carbaryl were obtained from Wako Pure Chemical Industries (Osaka, Japan). LPS from *Escherichia coli* O111:B4 was purchased from Sigma–Aldrich (St. Louis, MO). Anti-mouse iNOS antibody (M-19) and anti-actin antibody (C-11) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-sera against NF-κB p50 (#1157), p65 (#1226), IκB-α (#751), and IκB-β (#854) were kindly provided by Dr. Nancy Rice (NCI-Frederick Cancer Research and Development Center).

Plasmid construction. The mouse iNOS reporter plasmid, pGmiNF, was constructed by inserting a PCR-fragment of the mouse iNOS promoter region (−1588 to +161) into the *Mlu*I/*Xho*I site of pGL3-Basic vector (Promega, Madison, WI). The mouse IFN-β reporter plasmid, pmIFNβ-luc, was constructed by inserting a PCR-fragment of the mouse IFN-β promoter region (−716 to +27) into the *Mlu*I/*Xho*I site of pGL3-Basic vector. The NF-κB-dependent luciferase reporter plasmid, pELAM-S, was constructed by inserting a PCR-fragment of the E-selectin (ELAM-1) promoter [21] region (−170 to +52) into the *Sac*I/*Hin*dIII site of pGL3-Basic vector.

NO production. RAW 264 cells were plated ($1–5 \times 10^5$ cells/well) in 96-well plates and on the following day stimulated for 24 h. NO levels produced by cells were determined by measuring nitrite levels of the culture supernatants with Griess reagent [22].

RT-PCR. Preparation of total RNA from RAW 264 cells plated on 6-well plates was accomplished using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA (0.5 μg) was subjected to RT-PCR using the SuperScript one-step RT-PCR system with platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). Real-time RT-PCR was performed using Brilliant SYBR Green Single-Step QRT-PCR Master Mix (Stratagene, La Jolla, CA) on MX4000 Multiplex Quantitative PCR System (Stratagene). The following primer pairs were used: 5′-CTG CAG CAC TTG GAT CAG GAA CCT G-3′ (sense) and 5′-GGG AGT AGC CTG TGT GCA CCT GGA A-3′ (antisense) for detection of *iNOS* mRNA; 5′-CTC CAG CTC CAA GAA AGG ACG-3′ (sense) and 5′-GAA GTT TCT GGT AAG TCT TCG-3′ (antisense) for detection of *IFN-β* mRNA; 5′-ATC ACT GCC ACC CAG AAG ACT-3′ (sense) and 5′-TCC ACC ACC CTG TTG CTG TAG-3′ (antisense) for detection of *GAPDH* mRNA.

Reporter assay. The luciferase reporter assay was performed as described [23]. Briefly, RAW 264 cells ($3–5 \times 10^5$/well) were plated in 6-well plates and transfected on the following day using FuGene6 (Roche Diagnostics GmbH Mannheim, Germany) with either 0.5 μg pELAM-S, 0.5 μg pmIFNβ-luc or 1 μg pGmiNF together with 0.5 μg pSV-β-GAL (Promega) for normalization. At 24 h post-transfection, cells were stimulated for 6 h, and the reporter gene activity was measured according to the manufacturer's (Promega for luciferase assay, Clontech, Palo Alto, CA for β-galactosidase assay) instructions.

Preparation of cellular extracts and Western blotting. Cellular extracts were prepared as described [24,25] with slight modifications as follows. After washing with cold PBS, cells were lysed with 0.1 ml of a lysis buffer (10 mM Hepes–KOH, pH 7.9, 10 mM KCl, 5 mM EDTA, 40 mM β-glycerophosphate, 1% NP-40, 30 mM NaF, 1 mM Na_3VO_4, and 1 mM DTT) containing a protease inhibitor cocktail (Roche Diagnostics GmbH Mannheim, Germany). The lysates were centrifuged for 5 min at 1000*g* and the supernatants obtained were further centrifuged for 5 min at 12,000*g*. Obtained supernatants were used as cytosolic fractions. The pellets obtained at the first centrifugation were washed twice with the lysis buffer described above and nuclear proteins were extracted from the pellets for 10 min with 50 μl of an extraction buffer (20 mM Hepes–KOH, pH 7.9, 420 mM NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, and 25% glycerol). After vigorous mixing, the suspension was centrifuged (12,000*g*, 5 min) and the resulting supernatant was used as nuclear extracts. The protein concentrations were determined by the Bradford method, and the same amount of protein was loaded onto each lane of a discontinuous SDS–10% polyacrylamide gel (acrylamide/bisacrylamide ratio, 29:1) according to the method of Laemmli [26]. Proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA) and subjected to Western blotting with the indicated antibodies. The signals were visualized using an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ).

Results

Effects of alachlor and carbaryl on LPS-induced NO production

We previously reported that alachlor and carbaryl inhibited LPS-induced NO production by RAW 264 cells [20]. To clarify the mechanism of this inhibition, we first examined the concentration–response effects of alachlor and carbaryl on NO production. RAW 264 cells were treated with alachlor or carbaryl followed by LPS for 24 h, and the culture supernatants were harvested to quantify NO levels. Both chemicals inhibited the LPS-induced NO production in a concentration-dependent manner, and the inhibition was observed at concentrations as low as 1–10 μg/ml (Fig. 1).

Effects of alachlor and carbaryl on LPS-induced iNOS expression

Since NO produced by macrophages is catalyzed by the iNOS protein, we examined the effect of alachlor and carbaryl on the LPS-induced expression of the iNOS protein (Fig. 2A). In RAW 264 cells without stimulation, no iNOS protein was detectable utilizing Western blotting. However, LPS treatment did stimulate iNOS expression. When cells were treated with alachlor or carbaryl, these chemicals inhibited the LPS-induced expression of iNOS protein in a concentration-dependent manner, and 100 μg/ml of these chemicals completely inhibited the expression. The level of actin was

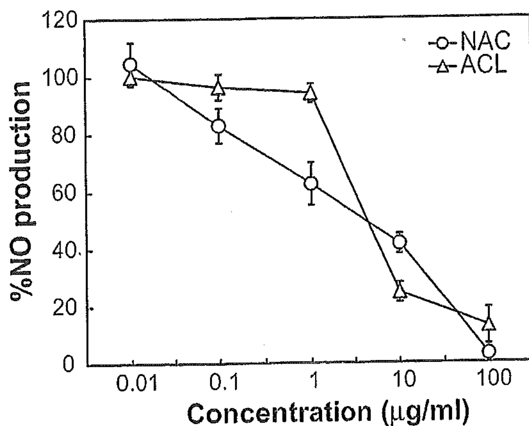

Fig. 1. Concentration-dependent effects of alachlor and carbaryl on LPS-induced NO production by RAW 264 cells. RAW 264 cells were stimulated with LPS (10 ng/ml) for 24 h with or without the indicated concentrations of alachlor (ACL) or carbaryl (NAC). The culture supernatants were analyzed for NO levels. NO production induced by LPS treatment alone is expressed as 100%.

not affected either by alachlor or carbaryl, indicating that these chemicals did not affect the cell viability.

It has been reported that the expression of iNOS in macrophages is mainly regulated at the transcriptional level [5,7,8]. Thus, we examined the expression of *iNOS* mRNA (Fig. 2B). RAW 264 cells were stimulated with LPS for 6 h and total RNA was prepared from these cells. Then, *iNOS* mRNA was amplified by RT-PCR. Without stimulation, no *iNOS* mRNA was detectable, however, LPS treatment stimulated its expression. When cells were treated with alachlor or carbaryl, these chemicals inhibited the LPS-induced expression of *iNOS* mRNA without affecting *GAPDH* mRNA levels. The iNOS promoter activity measured by transfecting RAW 264 cells with a luciferase reporter gene containing a mouse iNOS promoter was also inhibited by these chemicals at a similar concentration range (Fig. 2C). These results clearly indicate that alachlor and carbaryl inhibited LPS-induced transcriptional activation of *iNOS* mRNA expression.

Effects of alachlor and carbaryl on LPS-induced IFN-β expression

Since IFN-β is known to play an indispensable role in the LPS-induced expression of iNOS, it is possible that the reduction of LPS-induced iNOS expression in the presence of alachlor or carbaryl may be caused by a decrease in LPS-induced IFN-β production. To address this possibility, we examined the effect of alachlor and carbaryl on LPS-induced expression of IFN-β in RAW 264 cells. First, an RT-PCR analysis was performed using iNOS-specific primers. Without stimulation, *IFN-β* mRNA was not detectable; however, LPS treatment stimulated its expression. When cells were treated

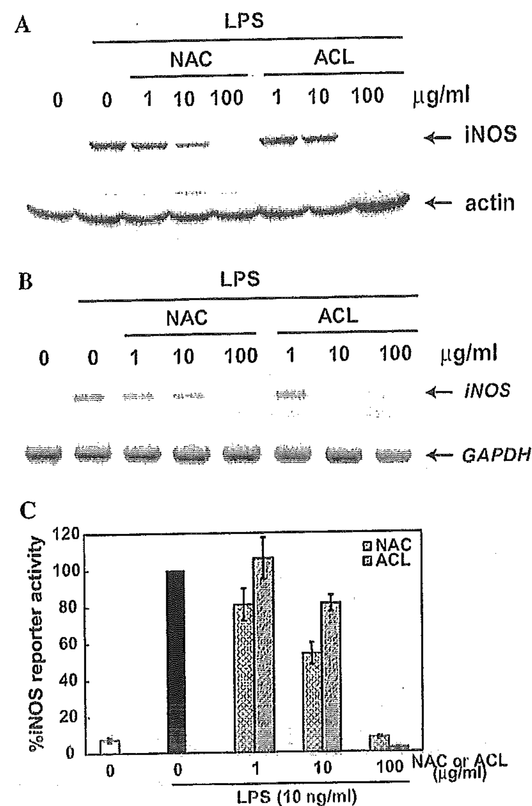

Fig. 2. Effects of alachlor and carbaryl on LPS-induced iNOS expression. RAW 264 cells were either left unstimulated or were stimulated with LPS (10 ng/ml) for 6 h with or without the indicated concentrations of alachlor (ACL) or carbaryl (NAC). Then the cytosolic fractions and total RNA were prepared from the cells and analyzed for iNOS and actin proteins by Western blotting (A), and for *iNOS* and *GAPDH* mRNA expression by RT-PCR (B). RAW 264 cells were transiently transfected with a luciferase reporter plasmid containing a mouse iNOS promoter together with pSV-β-GAL. After 24 h, cells were either left unstimulated or were stimulated for 6 h with LPS (10 ng/ml) with or without the indicated concentrations of alachlor (ACL) or carbaryl (NAC), and luciferase activity was then measured. Values are means ± SEM from three independent experiments. The reporter activity in response to LPS alone is expressed as 100%.

with alachlor or carbaryl, these chemicals inhibited the LPS-induced expression of *IFN-β* mRNA without affecting *GAPDH* mRNA levels (Fig. 3A). Quantitative RT-PCR also showed that alachlor and carbaryl inhibited the expression of *IFN-β* mRNA (data not shown). The IFN-β promoter activity, measured by transfecting RAW 264 cells with a luciferase reporter gene containing mouse IFN-β promoter, was also inhibited by these chemicals (Fig. 3B). These results indicate that alachlor and carbaryl inhibit LPS-induced transcriptional activation of *IFN-β* mRNA expression.

Previous results raised the possibility that alachlor and carbaryl suppress LPS-induced iNOS expression by inhibiting IFN-β production. To address this possibility, we examined the effect of exogenous IFN-β on the inhibitory effect of alachlor and carbaryl (Fig. 4).

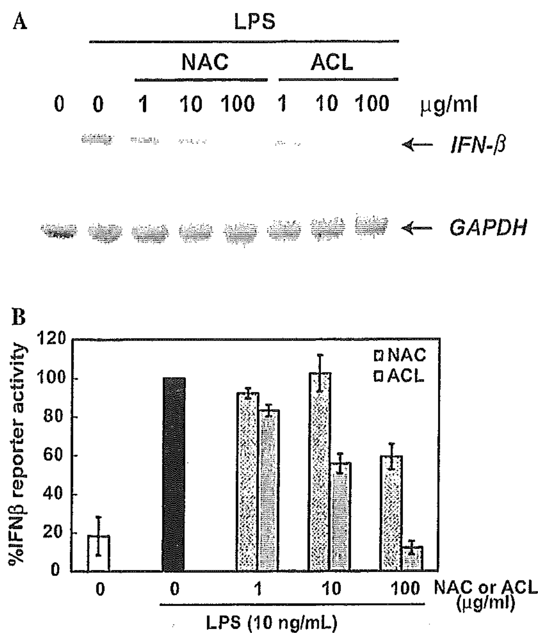

Fig. 3. Effects of alachlor and carbaryl on LPS-induced IFN-β expression. RAW 264 cells were either left unstimulated or were stimulated with LPS (10 ng/ml) for 6 h with or without the indicated concentrations of alachlor (ACL) or carbaryl (NAC). Then total RNA was prepared from the cells and analyzed for *iNOS* and *GAPDH* mRNA expressions by RT-PCR (A). RAW 264 cells were transiently transfected with a luciferase reporter plasmid containing a mouse IFN-β promoter together with pSV-β-GAL. After 24 h, cells were either left unstimulated or were stimulated for 6 h with LPS (10 ng/ml) with or without the indicated concentrations of alachlor (ACL) or carbaryl (NAC), and luciferase activity was then measured. Values are means ± SEM from six to seven independent experiments. The reporter activity in response to LPS alone is expressed as 100% (B).

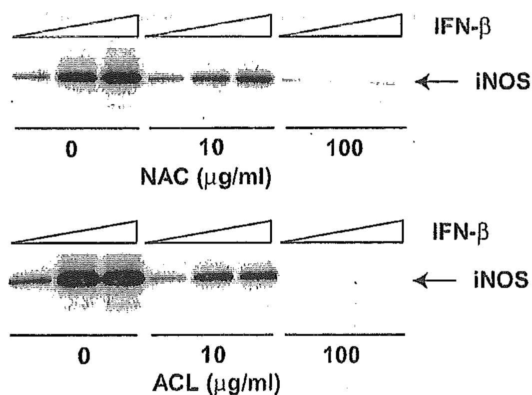

Fig. 4. Supplementation of IFN-β is not enough to restore the expression of iNOS after inhibition with alachlor and carbaryl. RAW 264 cells were stimulated with LPS (10 ng/ml) for 6 h with or without the indicated concentrations of alachlor (ACL) or carbaryl (NAC) together with increasing concentrations of recombinant IFN-β (0, 600, and 2000 U/ml). Then the cytosolic fractions were prepared from the cells and analyzed for iNOS protein by Western blotting.

RAW 264 cells were stimulated with LPS in the presence of 0–100 μg/ml of alachlor or carbaryl together with increasing concentrations of recombinant IFN-β, and iNOS protein levels were detected by Western blot. The LPS-induced expression of iNOS was significantly enhanced by exogenous IFN-β in the absence of alachlor or carbaryl. In the presence of 10 μg/ml alachlor or carbaryl, iNOS expression was inhibited to some extent and the inhibition was partially reversed by exogenous IFN-β. However, in the presence of 100 μg/ml alachlor or carbaryl, iNOS expression was completely inhibited by these chemicals and the inhibition was not reversed by exogenous IFN-β. This result suggests that the inhibition of LPS-induced iNOS expression by these chemicals is not explained solely by the inhibition of IFN-β production and that other factors may be involved.

Effects of alachlor and carbaryl on LPS-induced activation of NF-κB

The activation of NF-κB is known to be indispensable in the LPS-induced expression of both iNOS and IFN-β. In order to evaluate the involvement of factors other than the decrease in IFN-β production on the inhibitory effect of alachlor and carbaryl for LPS-induced expression of iNOS, we examined the effect of these chemicals on LPS-induced activation of NF-κB (Fig. 5). RAW 264 cells were transiently transfected with an NF-κB-dependent reporter plasmid, and then stimulated with LPS with or without alachlor or carbaryl treatment. The reporter activities were inhibited by these chemicals in a concentration-dependent manner, indicating that NF-κB is inhibited by alachlor and carbaryl.

To confirm the above finding, nuclear translocation of NF-κB subunits, p50 and p65, as well as the degradation of IκB-α and IκB-β in response to LPS-stimulation were examined by Western blot analysis (Fig. 6). RAW 264 cells were stimulated for the indicated time periods

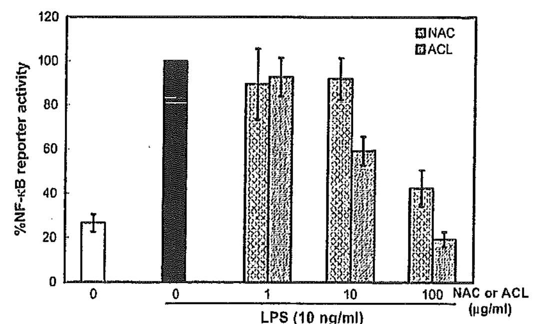

Fig. 5. Effects of alachlor and carbaryl on LPS-induced activation of NF-κB. RAW 264 cells were transiently transfected with an NF-κB-dependent luciferase reporter plasmid together with pSV-β-GAL. After 24 h, cells were either left unstimulated or were stimulated for 6 h with LPS (10 ng/ml) with or without the indicated concentrations of alachlor (ACL) or carbaryl (NAC), and luciferase activity was then measured. Values are means ± SEM from four independent experiments. The reporter activity in response to LPS alone is expressed as 100%.

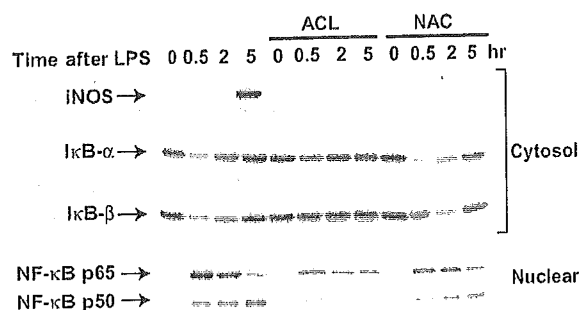

Fig. 6. Effects of alachlor and carbaryl on LPS-induced nuclear translocation of NF-κB and degradation of IκB proteins. RAW 264 cells were stimulated with LPS (10 ng/ml) for the indicated time periods with or without 100 μg/ml alachlor (ACL) or carbaryl (NAC) and cellular extracts were prepared. Cytosolic extracts were analyzed for iNOS, IκB-α, and IκB-β proteins, and nuclear extracts were analyzed for NF-κB p65 and p50 proteins by Western blotting.

with LPS with or without alachlor or carbaryl treatment. Cytosolic and nuclear proteins were then extracted for the detection. The expression of iNOS protein was observed in the cytosolic fraction at 5 h following LPS stimulation and was completely inhibited by alachlor and carbaryl as mentioned above. Preceding iNOS expression, accumulation of p65 and p50 subunits in the nuclear fraction was observed 30 min after LPS stimulation. This accumulation was also inhibited by alachlor and carbaryl. This result is consistent with the inhibition of the LPS-induced NF-κB reporter activity observed above. The degradation of IκB-α and IκB-β in response to LPS stimulation was observed to have similar time kinetics as the nuclear translocation of p65 and p50 subunits, but those levels gradually returned to the original levels due to re-synthesis. Alachlor treatment prevented the LPS-induced degradation of both IκB-α and IκB-β almost completely, whereas the degradation of these proteins was still observed and somewhat enhanced by carbaryl treatment. These results suggest that alachlor inhibits LPS-induced activation of NF-κB by preventing the degradation of IκB proteins, whereas carbaryl inhibits the activation of NF-κB by preventing its nuclear translocation.

Effects of reducing agents on the inhibitory activity of alachlor and carbaryl

Since both alachlor and carbaryl possess alkylating reactivity, we examined whether this reactivity is associated with the inhibitory effect of these chemicals on LPS-induced iNOS expression (Fig. 7). The LPS-induced expression of iNOS was completely inhibited by 100 μg/ml alachlor and carbaryl as described earlier. When both chemicals were pretreated with 1 mM dithiothreitol (DTT) or 2-mercaptoethanol (2-ME) before treatment of RAW 264 cells, the inhibitory effect of alachlor was not affected by these reducing agents, whereas

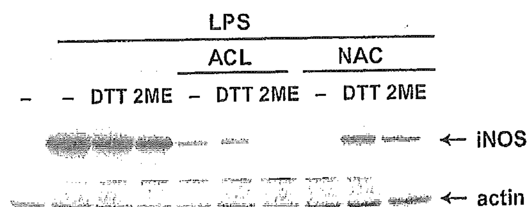

Fig. 7. Effects of reducing agents on the inhibitory effect of alachlor and carbaryl on LPS-induced iNOS expression. RAW 264 cells were either left unstimulated or were stimulated with LPS (10 ng/ml) for 6 h with or without 100 μg/ml alachlor (ACL) or carbaryl (NAC) that had been pretreated with 1 mM DTT or 2-ME or left untreated. Then the cytosol fractions were prepared from the cells and analyzed for iNOS and actin proteins by Western blotting.

the effect of carbaryl was weakened and only a slight inhibition was observed. This result suggests that the alkylating reactivity of carbaryl is involved in its inhibitory effect on LPS-induced iNOS expression and that alachlor and carbaryl utilize different mechanisms to inhibit LPS-induced iNOS expression.

Discussion

In the present study, we showed that LPS-induced iNOS expression in RAW 264 cells was inhibited by the treatment of alachlor or carbaryl, both of which are generally used as agrochemicals. These chemicals also inhibited LPS-induced *iNOS* mRNA expression and iNOS promoter activity, indicating that both chemicals inhibit iNOS expression at the transcriptional level. For transactivation of the *iNOS* gene, NF-κB and other transcription factors induced by type I IFN have been reported to be indispensable [5,9,10,12,13]. Thus, we investigated the effect of alachlor and carbaryl on LPS-induced expression of IFN-β and found that both chemicals inhibited this expression. However, the supplementation of IFN-β was not enough to restore the inhibition of iNOS expression caused by these chemicals. Since IFN-β by itself is not able to induce iNOS expression, we explored the involvement of other factors in the inhibition and found that both chemicals inhibited LPS-induced activation of NF-κB. NF-κB is known to be indispensable for LPS-induced expression of both IFN-β and iNOS [9,14–17]. It is, therefore, likely that the inhibition of LPS-induced NF-κB activation leads to the suppression of both iNOS and IFN-β expressions. Both alachlor and carbaryl inhibited LPS-induced nuclear translocation of p65 and p50, however, the site of action of these chemicals seems to be different. In the presence of alachlor, LPS-induced degradation of IκB-α and IκB-β proteins was inhibited, indicating that alachlor acts upstream of these processes. On the other hand, carbaryl did not inhibit degradation of these IκB proteins, indicating that carbaryl acts downstream

of this degradation, probably affecting the nuclear translocation process. Thus, these chemicals inhibit LPS-induced activation of NF-κB by different mechanisms.

Both alachlor [27] and carbaryl [28] are known to act as alkylating agents. Some alkylating chemicals have been reported to inhibit LPS-induced NO production by mouse macrophage cells, and this inhibitory effect was reversed by treating these chemicals with reducing agents [29,30]. Thus, we investigated the effect of reducing agents on the inhibitory action of these chemicals on LPS-induced iNOS expression. We found that DTT and 2-ME reversed the inhibitory activity of carbaryl but the activity of alachlor was not affected. This indicates that alkylating reactivity of carbaryl is involved in the inhibition of iNOS expression. This also suggests that the alkylation may affect the nuclear translocation process of p50 and p65 because carbaryl inhibited the translocation process (see above).

NO production by macrophages is important for host organisms protecting them from bacterial infections. In the present study, we provided evidence that two commonly used agrochemicals inhibit this macrophage function. It will be important, therefore, to investigate the influence of these chemicals on innate immunity.

Acknowledgments

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