Activation of the RON Receptor Tyrosine Kinase Inhibits Inducible Nitric Oxide Synthase (iNOS) Expression by Murine Peritoneal Exudate Macrophages: Phosphatidylinositol-3 Kinase Is Required for RON-Mediated Inhibition of iNOS Expression

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Activation of the RON Receptor Tyrosine Kinase Inhibits Inducible Nitric Oxide Synthase (iNOS) Expression by Murine Peritoneal Macrophages: Phosphatidylinositol-3 Kinase Is Required for RON-Mediated Inhibition of iNOS Expression

Yi-Qing Chen, James H. Fisher, and Ming-Hai Wang

RON (recepteur d’origine nantais) is a receptor tyrosine kinase expressed in murine peritoneal resident macrophages and activated by macrophage-stimulating protein (MSP). The objectives of this investigation were to study the RON expression in exudate macrophages and the mechanisms by which RON inhibits inducible nitric oxide synthase (iNOS) expression induced by LPS and IFN-γ. We found that mouse peritoneal resident and Con A-elicited macrophages collected on day 3 or day 5 express RON. Acute exudate macrophages collected on day 1 did not express RON. Activation of RON inhibited LPS- and IFN-γ-induced macrophage nitric oxide production and iNOS mRNA accumulation. Similar inhibition was observed also in Raw264.7 macrophage cell lines transfected with human RON cDNA. In these cells, MSP induced RON phosphorylation concomitant with reduced iNOS mRNA expression and protein synthesis. Further, we show that activated RON inhibited the iNOS gene transcription activity as assessed by chloramphenicol acetyltransferase activity in Raw264.7 cells expressing RON. Wortmannin, a specific inhibitor of phosphatidylinositol-3 (PI-3) kinase, prevented the inhibitory effect of RON on the iNOS gene promoter activity and on the nitric oxide production induced by LPS and IFN-γ. These effects were confirmed further by introducing a dominant-inhibitory PI-3 kinase p85 subunit in RON-expressing Rwa264.7 cells. Taken together, our results suggest that RON is expressed in peritoneal macrophages at later stages of inflammation. Activation of RON by MSP in mature exudate macrophages inhibits LPS- and IFN-γ-induced iNOS synthesis. PI-3 kinase is an important effector molecule required for RON-mediated inhibition of iNOS expression in macrophages. The Journal of Immunology, 1998, 161: 4950–4959.
macrophages (37, 38). Several studies indicate that RON expression in macrophages is regulated in monocyte differentiation and inflammation (37). Stimulation of RON-expressing cells with MSP rapidly induces tyrosine phosphorylation of RON (36, 37). Intra-cellular signaling proteins such as phosphatidylinositol-3 (PI-3) kinase, phospholipase C-γ, and mitogen-activated protein (MAP) kinase (Erk2) were also phosphorylated and activated upon MSP stimulation (39, 40), suggesting that RON is capable of stimulating diverging signaling pathways that may be involved in MSP-induced biologic activities in macrophages (39, 40). Recently, we have found that activation of RON by MSP in murine peritoneal resident macrophages results in inhibition of iNOS expression induced by LPS and proinflammatory cytokines, including IFN-γ and TNF-α (22). The mechanism for such inhibition is currently unknown. Studies using RON knockout mice showed that inactivation of the RON gene increases the susceptibility to LPS-induced septic shock (41). This effect was accompanied by increased NO production by RON−/− macrophages (41). Therefore, RON plays a critical role in regulating macrophage activities in response to inflammatory stimulation (22, 41).

In the present report, we have studied the expression of RON in peritoneal exudate macrophages and the role of RON in regulation of LPS-induced NO production. Also, we have established Raw264.7 cell lines expressing human RON and studied the mechanisms by which RON activation inhibits LPS-induced iNOS expression. Our results showed that MSP inhibits iNOS expression in both resident macrophages and mature exudate macrophages. RON activation by MSP transduces inhibitory signals that block LPS- and IFN-γ-induced iNOS promoter activity. The specific PI-3 kinase inhibitor wortmannin and a dominant-inhibitory p85 of PI-3 kinase prevent the inhibitory effect of RON, suggesting that RON-induced activation of PI-3 kinase is an important intracellular component that transduces inhibitory signals in MSP-treated macrophages.

Materials and Methods

Cells

The murine macrophage-like cell line Raw264.7 was obtained from American Type Culture Collection (Manassas, VA) and adapted to macrophage serum-containing medium (M-SFM, Life Technologies, Gaithersburg, MD). Mouse peritoneal resident macrophages were obtained from C3H/HeN mice by lavage of the peritoneal cavity with 10 ml of RPMI 1640 containing 1% fetal bovine serum. Peritoneal exudate macrophages were collected from mice at different intervals after i.p. injection of 1 ml of Con A solution (40 mg in PBS, Sigma, St. Louis, MO). Collected cells were washed twice with cold RPMI 1640 at 4°C. All cell cultures were maintained in M-SFM at 37°C in a humidified incubator containing 5% CO2 in air.

Reagents

Purified human plasma MSP (19) was kindly provided by Dr. E. J. Leonard (National Cancer Institute, Frederick, MD), Rat polyclonal Abs to synthetic C-terminal peptide of human or mouse RON were generated from rabbits immunized with keyhole limpet hemocyanin-conjugated peptides. An mAb to human RON (clone ID2) was used as described (39). Mouse rabbits immunized with keyhole limpet hemocyanin-conjugated peptides. Purified human plasma MSP (19) was kindly provided by Dr. E. J. Leonard (20). The mouse iNOS cDNA (43) was provided by Dr. C. Lowenstein (The Johns Hopkins University, Baltimore, MD). The 5.0-kb DNA fragment containing the mouse iNOS gene promoter (44) was provided by Dr. H. Esumi (National Cancer Center, Tokyo, Japan). The cDNA was inserted into the vector pBLCAT5 to generate the reporter construct pNOSCAT1 (44). LPS prepared from Escherichia coli serotype 055:B5 was from Life Technologies. Wortmannin was from Calbiochem (San Diego, CA). Sulfanilamide, naphthylethylenediamine dihydrochloride, sodium nitrite, and other chemicals were from Sigma.

Establishment of Raw264.7 cell lines expressing human RON

Transfection of Raw264.7 cells with an expression vector pDR2 containing a full length of human RON cDNA was performed using electroporation (44). Briefly, cells (1×107) were mixed with 5 μg of the plasmid in 0.5 ml of PBS, pH 7.6, and electroporated at 330 V and 500 μF. Recovered cells were incubated in M-SFM at 37°C for 24 h and then switched to fresh medium containing 400 μg/ml of hygromycin B. The colonies were picked and expanded into cell lines. Expression of RON by individual clones was determined by Western blotting using rabbit IgG to RON after immunoprecipitation. Two cell lines expressing high levels of RON were obtained and designated as RawRON8 and RawRON32.

Expression of a dominant-inhibitory p85 of PI-3 kinase (Δp85) in Raw264.7 cells expressing human RON

RawRON8 cells were transfected with 5 μg of pcDNA3 containing the cDNA encoding Δp85 using transfection reagent N-[1-(2,3-dioleoyloxy)propyl]-N,N,N,N-tetramethylammonium methyl sulfate (DOTAP; Boehringer Mannheim) as described previously (20). Transfected cells were selected in 400 μg/ml of G418. Colonies were picked and expanded into cell lines. Expression of Δp85 in each cell line was determined by Western blotting using rabbit IgG specific to p85 (Transduction Laboratories, Lexington, KY). Two cell clones, Δp85−1 and Δp85−2, expressing different levels of Δp85 were used in functional studies.

Assay for NO2− production

Macrophages at 2×105 cells/ml were incubated in M-SFM in 200 μl/well in a 96-well Microtiter plate. Cells were stimulated with LPS, IFN-γ, or their combinations in the presence or absence of different concentrations of MSP. Cultured fluids were collected 36 h after incubation. Synthesis of NO was determined by measuring NO2−, a stable reaction product of NO with molecular oxygen, using Griess reagent (0.05% sulfanilamide and 0.05% N-(1-naphthyl) ethylenediamine dihydrochloride in 2.5% H3PO4) as previously described (22). The optical densities of each sample were measured by an ELISA reader at the wavelength of 570 nm. NO2− concentrations were calculated by comparison with a standard curve prepared using NaNO2.

Immunoprecipitation and Western blotting

These procedures were performed as described previously (36). Briefly, macrophages were lysed in 0.5 ml of lysis buffer (0.1 M Tris buffer, pH 7.6, containing 0.15 M NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.5% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 20 μg/ml soybean trypsin inhibitor, and 100 μM sodium vanadate). Lysates were centrifuged in a microcentrifuge, diluted with TBS-T buffer (100 mM Tris buffer, pH 7.6, containing 150 mM NaCl, 0.5% BSA, and 0.05% Tween-20), and precleared with normal mouse or rabbit IgG bound to protein G-Sepharose beads. Cellular proteins were immunoprecipitated with specific Abs bound to protein G-Sepharose beads. Samples were then solubilized and reduced in 100°C in sample buffer containing 2-ME and separated in 8% acrylamide gel containing SDS. After electrophoresis, proteins were transferred to an Immobilon-P membrane (Millipore, Bedford, MA). Rabbit IgG to human or mouse RON peptide, mAb 4G10 to phosphotyrosine (Upstate Biotechnology, Lake Placid, NY), or rabbit IgG to p85, respectively, was used to detect specific proteins. Goat anti-mouse or rabbit IgG conjugated with peroxidase (Boehringer Mannheim) were used as secondary Ab. The reaction was developed with enhanced chemiluminescent reagent (Amer sham, Arlington Heights, IL) and exposed to film. In some instances, membranes were treated with SDS/2-ME erosion buffer (20) and then reprobed with other Abs.

RNA isolation and Northern blot analysis

Total RNA was extracted from cultured macrophages using the RNAzol B (Biotex Laboratories, Houston, TX) according to the manufacturer’s protocol. All RNA samples had an A260/A280 ratio of >1.75. Aliquots containing 2 μg of total RNA were size fractionated in a 1% agarose gel containing 2.2 M formaldehyde. After electrophoresis, RNA was transferred to a nylon membrane (Boehringer Mannheim) and UV cross-linked. The hybridization procedures were conducted as described previously (22). The mouse iNOS cDNA fragment labeled with [32P]dCTP (Amersham) was used as a specific probe. The β-actin cDNA was used as control. The radioactivity on the membrane was autoradiographed at ~85°C on XAR-5 film (Eastman Kodak, Rochester, NY) with the use of intensifying screens (DuPont, Wilmington, DE).
Chloramphenicol acetyltransferase (CAT) assay for detection of iNOS promoter activity

The method has been described previously (45). Briefly, RON-expressing Raw264.7 or control cells were cultured in M-SFM. For transient transfection analysis, cells at 50% confluence in a 6-mm culture dish were transfected with 10 μg of the pNOSCAT1 using the tranfection reagent DOTAP as described (31). The β-galactosidase reference plasmid pRSVβgal was used as an internal standard for transfection efficiency. After 24 h, cells were washed once with M-SFM and then stimulated with LPS, IFN-γ, or their combinations in the presence or absence of MSP. After incubation for an additional 40 h, whole cell extracts were prepared by disrupting cells with three freeze-thaw cycles. The supernatants were assayed for the amounts of CAT proteins using a CAT-specific ELISA kit (5-Primer–3-Primer, Boulder, CO). Experiments were repeated three times. The concentrations of the CAT protein were normalized for β-galactosidase activity.

Results

Expression of RON in Con A-elicited peritoneal exudate macrophages

Expression of RON in murine resident peritoneal macrophages has been reported previously (37). However, it is unclear whether inflammatory macrophages express RON. To test this, peritoneal exudate macrophages were collected at several time intervals after Con A injection. Western blot analysis was conducted to determine RON expression. The results shown in Figure 1A demonstrated that resident peritoneal macrophages express RON. RON was not detected in acute inflammatory macrophages collected on day 1 (lane E1) but was expressed in exudate macrophages harvested on day 3 (lane E3) and day 5 (lane E5). The level of RON from exudate macrophages collected on day 5 is comparable with those from resident macrophages. We then tested whether MSP induces phosphorylation of RON in inflammatory macrophages. The results presented in Figure 1B show that MSP induced tyrosine phosphorylation of RON both in mature exudate and resident macrophages. The time course of RON phosphorylation induced by MSP in mature exudate macrophages is shown in Figure 1C. The phosphorylated RON was first detected 5 min after MSP stimulation, peaked at 15 min, and then gradually reduced to a lower level.

Inhibition of iNOS expression in mature exudate macrophages by MSP

To determine whether RON activation affects the production of NO by activated macrophages, Con A-elicited mouse peritoneal macrophages were collected at different time intervals and analyzed for the effect of MSP on LPS- and IFN-γ-induced NO production. The results are shown in Table I. Consistent with our previous report (22), we showed that MSP inhibited LPS- or LPS plus IFN-γ-induced NO production by resident macrophages. MSP had no effect on LPS- and IFN-γ-induced NO formation by acute exudate macrophages collected on day 1. In contrast, exudate macrophages collected on day 3 responded to MSP. Accumulation of NO in culture fluids was significantly reduced in the presence of MSP, with inhibition ranging from 72% in LPS alone to 76% in LPS- and IFN-γ-treated cells. These data indicate that RON activation inhibits LPS- and IFN-γ-stimulated NO production not only in resident cells, but also in Con A-elicited mature exudate macrophages.

The effect of RON activation on the inhibition of iNOS expression was studied further at the protein and mRNA levels. We also compared the inhibitory effect of MSP with other iNOS inhibitors, such as TGF-β and IL-4. The effect of MSP on expression of the iNOS protein is shown in Figure 2A. The amounts of the induced iNOS protein in MSP-treated cells were dramatically diminished when compared with cells stimulated with LPS. Also, these results show that MSP inhibited not only LPS-induced iNOS protein synthesis, but also the synergistic effect of LPS and IFN-γ on the iNOS protein expression. In addition, these results show that the inhibitory effect of MSP is comparable with that of TGF-β and is much stronger than that of IL-4. IL-4 only partially inhibited the LPS- and IFN-γ-induced iNOS protein expression. The Northern blot analysis of the iNOS mRNA expression is shown in Figure 2B. MSP inhibited the iNOS mRNA expression induced by LPS.
Table I. Effect of MSP on LPS and IFN-γ-induced NO production by murine peritoneal resident and exudate macrophagesa

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>Resident</th>
<th>Acute exudate (day 1)</th>
<th>Mature exudate (day 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>1.8 ± 0.4</td>
<td>3.5 ± 1.6</td>
<td>1.9 ± 1.4</td>
</tr>
<tr>
<td>MSP</td>
<td>1.9 ± 0.6</td>
<td>3.1 ± 0.3</td>
<td>2.8 ± 1.9</td>
</tr>
<tr>
<td>LPS</td>
<td>28.4 ± 4.3</td>
<td>33.7 ± 3.9</td>
<td>28.7 ± 3.7</td>
</tr>
<tr>
<td>LPS + MSP</td>
<td>4.6 ± 2.1</td>
<td>32.5 ± 3.8</td>
<td>8.1 ± 2.2</td>
</tr>
<tr>
<td>LPS + IFN-γ</td>
<td>39.2 ± 3.4</td>
<td>41.5 ± 3.8</td>
<td>38.6 ± 4.4</td>
</tr>
<tr>
<td>LPS + IFN-γ + MSP</td>
<td>7.4 ± 4.6</td>
<td>40.7 ± 3.2</td>
<td>6.9 ± 3.5</td>
</tr>
</tbody>
</table>

a Exudate macrophages were collected after Con A injection. Resident cells were used as control. Cells (1 × 10⁶/well) were stimulated with LPS (1 µg/ml) or together with IFN-γ (100 U/ml) for 24 h. MSP (5 nM) was used simultaneously. Nitrite in culture fluids was determined by Griess reaction. Data are shown as mean ± SD of triplicate experiments. Similar results were obtained from two other experiments.

FIGURE 2. Effect of MSP on LPS- and IFN-γ-induced iNOS mRNA or protein expression by mouse peritoneal exudate macrophages. Peritoneal exudate macrophages were collected 3 days after i.p. injection of Con A. Cells (1 × 10⁶ cells/culture dish) were stimulated with 1 µg/ml of LPS or with 100 U/ml of mouse IFN-γ in the presence of different concentrations of MSP. TGF-β (10 ng/ml) and IL-4 (20 ng/ml) were also included in the experiments. A. Inhibition of LPS- and IFN-γ-induced expression of the iNOS protein by MSP. Western blot analysis was performed using cell lysates prepared 10 h after stimulation. Rabbit IgG to mouse iNOS was used as detecting Ab. Lane 1, cell control; lane 2, LPS; lane 3, LPS + IFN-γ; lane 4, LPS + MSP (5 nM); lane 5, LPS + IFN-γ + MSP (10 nM); lane 6, LPS + IFN-γ + IL-4; lane 7, LPS + IFN-γ + TGF-β. B. Dose-dependent inhibition of iNOS mRNA expression by MSP. Northern blot analysis was conducted using total RNA prepared 6 h after stimulation. The 32P-labeled mouse iNOS cDNA fragment was used as a probe. Lane 1, cell control; lane 2, MSP (5 nM); lane 3, LPS + IFN-γ; lane 4, LPS + IFN-γ with TGF-β; lanes 5 to 7, LPS + IFN-γ with MSP (0.1, 1, and 10 nM, respectively). C. Same membrane was reprobed with β-actin cDNA to show the loading of total RNA.

and IFN-γ. This effect was dose dependent. At 10 nM, MSP inhibited the iNOS mRNA expression by about 80%. These data suggest that the effect of MSP on LPS-induced NO production by mature exudate macrophages is targeted at the level of the iNOS mRNA accumulation.

Activation of RON in Raw264.7 cells inhibits LPS- and IFN-γ-induced iNOS synthesis

To study the effect of RON-mediated inhibition of iNOS expression in more detail, we transfected Raw264.7 cells with human RON cDNA by electroporation. Raw264.7 cells do not express endogenous RON as determined by Northern and Western blot analysis (data not shown). Also, MSP has no effect on LPS or LPS plus IFN-γ-induced NO production by Raw264.7 cells (our unpublished data). By cDNA transfection, two cell clones, namely RawRON8 and RawRON32, expressing high levels of RON were obtained and expanded into cell lines. Results presented in Figure 3A show the expression of RON in RawRON8 and RawRON32, as detected by Western blot after immunoprecipitation of RON with mAb ID2. An unprocessed, single-chain RON precursor (pro-RON) with a molecular mass of 180 kDa was observed. About half of the expressed protein is processed into the mature RON αβ-chain heterodimer. This is evident by the presence of the 145-kDa RON β-chain. The 40-kDa α-chain is not shown, because our rabbit IgG only recognizes the C-terminal peptide of the RON β-chain. In Figure 3B, we show that stimulation of these cells with...
MSP induced tyrosine phosphorylation of RON, indicating that the expressed RON is functional.

After confirming the RON expression, we tested whether the inhibitory effect of MSP could be reproduced by using Raw264.7 cells expressing human RON. To this end, RawRON8 cells were stimulated with LPS in the presence or absence of MSP. The inhibitory effect of MSP was compared also with other iNOS inhibitors, such as IL-4, IL-10, and TGF-β. Expression of the iNOS protein was evaluated by Western blotting. As shown in Figure 4A, LPS strongly induced expression of the iNOS protein in RawRON8 cells. MSP totally blocked expression of the iNOS protein induced by LPS, and its effect was comparable with that of TGF-β.

IL-10 had no effect on LPS-induced synthesis of the iNOS protein. IL-4 only partially antagonized the LPS effect. Similar data were obtained with RawRON32 cells (our unpublished data). To determine whether the MSP affects the iNOS mRNA expression, RawRON8 cells were treated with LPS and IFN-γ. Different amounts of MSP were added simultaneously after initiation of cell culture. The results shown in Figure 4B demonstrate the dose-dependent inhibition of iNOS mRNA expression by MSP. Again, MSP at 10 nM could totally block the synergistic effect of LPS and IFN-γ on iNOS gene expression. These results are similar to those shown in Figure 2B, indicating that the inhibitory effect of MSP could be reproduced in Raw264.7 cells expressing human RON, and that the action of MSP is targeted at the level of the iNOS mRNA expression.

Inhibition by MSP of the promoter activity of the iNOS gene in RON-transfected Raw264.7 cells

To explore the possibility that MSP inhibits iNOS transcription, we transiently transfected RawRON8 cells with the piNOSCAT1 reporter gene construct (44). After stimulation of cells with LPS alone or combined with IFN-γ, the effect of MSP on the iNOS promoter activity was determined by the CAT assay. The results presented in Figure 5 show that in cells stimulated with LPS and IFN-γ, the amounts of CAT were significantly increased in comparison with cells without treatment, indicating that LPS and IFN-γ activated the iNOS promoter. MSP had no effect by itself on iNOS promoter activity but significantly inhibited LPS-stimulated CAT production. MSP also inhibited the synergistic effect of LPS and IFN-γ on CAT production. These data suggest that MSP-mediated inhibition of iNOS transcription is mediated by inhibiting the promoter activity of the iNOS gene activated by LPS and IFN-γ. In addition, MSP did not inhibit the LPS-induced promoter activity of the iNOS gene in parental (untransfected) Rwa264.7 cells (our unpublished data).

FIGURE 4. Inhibition by MSP of LPS- and IFN-γ-induced iNOS synthesis in RON-expressing Raw264.7 cells. RawRON8 cells (× 10⁶ cells/culture dish) were stimulated with LPS (1 µg/ml) or LPS plus IFN-γ in the presence or absence of MSP. Experimental conditions were similar to those described in Figure 2. A, Detection of the iNOS protein by Western blotting. Lane 1, medium; lane 2, LPS; lanes 3 to 6, LPS with IL-10 (10 ng/ml), TGF-β (10 ng/ml), IL-4 (20 ng/ml), and MSP (10 nM), respectively. Lane P, positive control for iNOS. B, Northern blot analysis of the iNOS mRNA expression. Lane 1, cell control; lane 2, LPS + IFN-γ; lanes 3, 4, and 5, LPS + IFN-γ with 10, 1, and 0.1 nM MSP, respectively. C, The same membrane was reprobed with β-actin cDNA to show the loading of the RNA samples.

FIGURE 5. Inhibition of LPS- and IFN-γ-induced iNOS promoter activity by MSP. Transfection of RawRON8 cells with piNOSCAT1 was performed as described in Materials and Methods. After transfection, cells were stimulated with LPS (1 µg/ml) alone or with IFN-γ (500 U/ml). MSP (5 nM) was added simultaneously to cell culture. The amounts of CAT protein produced were quantified by an ELISA (48) and normalized for β-galactosidase activity. In each experiment, the relative amount of CAT induced by LPS was set at 100. Data are shown as means of triplicate (SD, <15%). Data are from one of three experiments with similar results.
Effect of wortmannin on RON-mediated inhibition of iNOS promoter activity

To determine whether the inhibitory effect of RON on iNOS promoter could be regulated by specific inhibitors such as wortmannin for PI-3 kinase (46), RON-expressing Raw264.7 cells were pretreated with wortmannin after transient transfection of pNOSCAT1. Cells were then treated with LPS to induce CAT reporter gene transcription. The results in Figure 6 show that preincubation of RawRON8 cells with 50 nM wortmannin had no effect on LPS-induced CAT production. However, wortmannin significantly increased the amounts of LPS-induced CAT proteins in cells treated with MSP, indicating that wortmannin prevents the inhibitory effect of MSP on the LPS-induced promoter activity of the iNOS gene.

Prevention of MSP-induced inhibition of NO production by wortmannin

Because wortmannin reduces the inhibitory effect of MSP on the LPS-induced transcription activity of the iNOS gene promoter, we wanted to determine whether it could prevent the MSP-induced inhibition of NO production. To test this hypothesis, RawRON8 cells were pretreated with wortmannin and then stimulated with LPS and IFN-γ. The amount of nitrite in culture fluid was determined by Griess reaction. The results presented in Figure 6 show that pretreatment of Rwa264.7 cells with wortmannin had no effect on LPS-induced CAT production. However, wortmannin significantly increased the amounts of LPS-induced CAT proteins in cells treated with MSP, indicating that wortmannin prevents the inhibitory effect of MSP on the LPS-induced promoter activity of the iNOS gene.

Effect of the dominant-inhibitory p85 of PI-3 kinase on RON-mediated iNOS inhibition

To investigate further whether activation of PI-3 kinase is required for the RON-mediated inhibition of iNOS expression, RawRON8 cells were introduced with an expression vector containing the dominant-inhibitory Δp85 of PI-3 kinase. The Δp85 has been used effectively to block endogenous PI-3 kinase activity (42). Transfected cells expressing Δp85 were obtained. The results in Figure 8A show the levels of Δp85 expressed by three individual clones. Using these cells, together with RawRON8 cells, the effect of Δp85 on MSP-induced inhibition of NO production was tested. The results in Figure 8B show that in LPS- and IFN-γ-stimulated RON-expressing Raw264.7 cells, MSP-mediated inhibition of NO production was prevented significantly by wortmannin. Similarly, in RawRON8 cells expressing Δp85, the inhibitory effect of MSP was significantly reduced. The levels of MSP-mediated NO inhibition were reduced to about 35% (clone 1) and 18% (clone 2) in these two cell lines, suggesting that inhibition of the endogenous PI-3 kinase by Δp85 prevented the MSP-mediated inhibition of LPS- and IFN-γ-induced NO production. Taken together, these data indicate that MSP-induced activation of PI-3 kinase mediates RON-induced inhibition of iNOS synthesis.

Discussion

The purpose of this investigation was to determine the role of RON in regulation of iNOS expression in peritoneal macrophages and to study the mechanisms by which RON activation inhibits macrophage iNOS expression induced by LPS and inflammatory cytokines such as IFN-γ. MSP inhibition of iNOS synthesis was first described in LPS- and cytokine-stimulated murine resident peritoneal macrophages (22). Physiologic concentrations of MSP induced a long lasting inhibitory effect on stimulated iNOS expression (22). MSP also blocked the iNOS mRNA expression induced by different iNOS inducers, including LPS, IFN-γ, TNF-α, IL-2, or their different combinations (22). We found that the inhibitory effect of MSP was specific for iNOS, because LPS-induced cytokine production such as monocyte chemoattractant protein 1 was not affected by MSP (22). The data presented here show that the action of MSP is not limited to resident macrophages. Exudate peritoneal macrophages 3 days after Con A injection express RON and respond to MSP. In contrast, acute exudate macrophages do not express RON. This effect can be reproduced by expression of...
RON in the established mouse macrophage-like cell line Raw264.7.

Several studies, including ours, have indicated that expression of RON is restricted to certain types of tissue macrophages (37, 38) and is regulated during inflammation (37). Mouse resident peritoneal macrophages express RON and respond to MSP with increased chemotactic migration, cell shape change, and phagocytosis (19). Osteoclasts, a member of the mononuclear phagocyte system, also express RON (47), and MSP stimulation facilitates the bone resorption activity of osteoclasts in vitro (47). In contrast, macrophages derived from alveolus, spleen, and bone marrow do not express RON and respond to MSP (47). These studies imply that RON expression is regulated such that not all populations of tissue-derived macrophages express RON. The restricted expression pattern also suggests that the transcription of the RON gene is controlled by the environment in which macrophages reside (37, 47). Iwama et al. (37) have presented evidence showing that expression of RON by peritoneal resident macrophages is related to macrophage differentiation in peritoneal cavity. Using a specific RON Ab and a marker Ab for macrophage, they demonstrated that blood monocytes do not express RON but gradually become predominantly RON positive several days after entrance into the peritoneal cavity (37). Based on these data, it was suggested that terminal differentiation of monocytes/macrophages in the peritoneal environment results in the expression of RON (37). We show that acute exudate peritoneal macrophages collected 1 day after induction of peritonitis by Con A do not express RON. RON is expressed in mature exudate macrophages 3 or 5 days after Con A stimulation, suggesting that RON is expressed by peritoneal macrophages at the later stage of inflammation. The mechanisms that regulate the RON expression in different types of tissue macrophages are unknown. Inflammatory mediators, such as cytokines, have been suggested to be involved in regulation of RON expression (48). Recent in vitro studies have demonstrated that human
monocyte cell line THP1 could be induced to express RON mRNA during cell differentiation into macrophages (48). Inflammatory cytokines such as IFN-γ and TNF-α facilitate the RON expression (48). Considering the promoter structures of RON that contain several cytokine-responsive elements (49), it is possible that inflammatory cytokines released during inflammation stimulate the RON gene transcription by inflammatory macrophages. Thus, it will be of interest to determine which cytokines or growth factors are capable of regulating RON expression in primary macrophages.

The ability of RON to inhibit LPS- and IFN-γ-induced iNOS expression in both resident and mature exudate macrophages suggests that RON activation may play a role in regulating macrophage activities during inflammation. Because RON is restricted to certain types of tissue macrophages, the differences in RON-positive and -negative macrophages may account for differences in susceptibility of specific tissues to injuries during inflammation. Our early studies have shown that activation of RON by MSP induces stimulatory activities in resident macrophages, including cell shape change, chemotactic migration to C5a, and phagocytosis of C3bi-coated erythrocytes (19). These observations imply that MSP may be involved in inflammation by activating tissue macrophages at the site of injury and stimulating their phagocytic activities. We have also observed increased ingestion of C3bi-coated Listeria monocytogenes by macrophages treated with MSP (31) and increased expression of RON in macrophages recruited into the peritoneal cavity in Con A-induced peritonitis. However, MSP inhibits LPS- and inflammatory cytokine-induced iNOS gene expression (Ref. 22 and present data). This activity is of particular interest, because it indicates that RON has dual functions in regulating macrophage activities during inflammation. Since excessive formation of NO is a major cause for tissue damage and septic shock (11–14), it is reasonable to speculate that blocking NO production by MSP might attenuate toxic effects of NO and regulate inflammatory responses. Recent in vivo studies using mice with the disrupted RON gene (knockout) have demonstrated that inactivation of RON significantly increased LPS- or IFN-γ-induced NO production by peritoneal macrophages and caused severe inflammatory reactions in delayed-type hypersensitivity (41). RON-deficient mice were also more susceptible to death induced by LPS at a dose that is sublethal to wild-type mice (41). These data suggest that RON expression can mediate outcome in LPS-induced septic shock. Although the causes of the increased inflammation in RON knockout mice are not known, it was suggested that lack of RON-mediated inhibition of macrophage iNOS synthesis is at least partially responsible for these pathophysiologic effects (41). Considering our present data showing that RON activation inhibits iNOS expression by inflammatory macrophages, the lack of such inhibition could be one of the mechanisms that lead to increased inflammation in vivo.

Many cytokines or growth factors have been found to inhibit macrophage iNOS expression through different mechanisms (16–18, 50). TGF-β inhibits iNOS expression by decreasing iNOS mRNA stability without affecting the transcription of the iNOS gene (16). IL-11 blocks iNOS expression by inhibition of NF-κB-dependent transcriptional activation (50). Evidence from this study indicates that the inhibition of NO production by MSP occurs at the transcriptional level. Activation of RON down-regulates LPS- and IFN-γ-induced expression of the iNOS mRNA in resident and mature exudate macrophage in a dose-dependent manner. Expression of iNOS mRNA by Raw264.7 cells expressing human RON was also inhibited. The inhibition of mRNA accumulation correlates with reduction of the iNOS protein expression and NO production by these cells. Moreover, these inhibitory effects can be...
reproduced in Raw264.7 cells expressing human RON. This notion is further supported by evidence from the studies of the iNOS gene promoter activity. RON activation inhibited LPS- and IFN-γ-induced iNOS gene promoter activity, indicating that the action of RON is targeted at the transcription level. However, since we have not studied the rate of the iNOS gene transcription and the mRNA stability, other inhibitory mechanisms cannot be excluded.

PI-3 kinase is a critical effector molecule involved in the RON-mediated signaling pathway that leads to inhibition of iNOS expression. Various molecular targets for PI-3 kinase have been identified. Among them are cytoskeletal proteins, protein kinases, and transcription factors (51, 52). Activated PI-3 kinase regulates cell growth, migration, and morphologic changes (51, 52). Thus, PI-3 kinase is regarded as an important regulator involved in many physiologic processes (51, 52). MSP-activated induction of PI-3 kinase has been reported in epithelial cells as well as in murine peritoneal resident macrophages (39). In human epithelial cells, MSP stimulates tyrosine phosphorylation of PI-3 kinase p85 subunit and induces association of RON with PI-3 kinase (39). These effects are believed to account for the ability of MSP to induce cell migration and morphologic change (39). Recently, the role of PI-3 kinase in regulation of iNOS expression has been documented (53, 54). Wright et al. (53) reported that IL-13-induced inhibition of iNOS expression in cytokine-stimulated HT-29 colonic epithelial cells was reversed by LY294002, a specific inhibitor for PI-3 kinase (53). Their results demonstrated that the activation of PI-3 kinase by IL-13 is a key signal that is responsible for the inhibition of iNOS transcription in activated epithelial cells (53). The effect of wortmannin on LPS-activated NO production by murine peritoneal macrophages was also reported (54). It was shown that wortmannin directly enhances LPS-induced NO production, suggesting that PI-3 kinase plays an important role in transducing the signals that are involved in LPS-stimulated macrophage activation (54). Consistent with these studies, our data show that activation of PI-3 kinase is required for RON-mediated iNOS inhibition. Three lines of evidence support this conclusion. First, wortmannin at a nanomolar concentration prevented the MSP-induced inhibition of iNOS gene promoter activity in LPS-stimulated RawRON8 macrophages. Second, wortmannin reversed the MSP-induced inhibitory effect on NO production stimulated by LPS and IFN-γ. Third, the expression of the dominant-inhibitory p85 of PI-3 kinase, which inhibits the endogenous PI-3 kinase (42), significantly blocked the RON-mediated inhibition of NO production. These data strongly suggest that the inhibition of MSP-activated activation of PI-3 kinase blocks the RON-transduced iNOS inhibitory signals. We speculate that lipid products of activated PI-3 kinase may inhibit LPS or IFN-γ-stimulated signal molecules or transcription factors that are essential for iNOS synthesis. However, because RON is capable of activating other signaling pathways, including the MAP kinase cascade (40), other signaling proteins, such as MAP kinase 1 (MEK1), may be involved in RON-mediated inhibition of LPS-induced iNOS expression as well. Indeed, our recent studies have found that the inhibitory effect of MSP on iNOS expression can be increased further by the presence of PD98059, a specific inhibitor of MEK1 (55). Studies are currently underway to determine the role of MEK1 in RON-mediated inhibition of iNOS expression in LPS- and IFN-γ-stimulated macrophages.

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