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The Inhibitory Action of Sodium Arsenite on Lipopolysaccharide-Induced Nitric Oxide Production in RAW 267.4 Macrophage Cells: A Role of Raf-1 in Lipopolysaccharide Signaling¹

Dipshikha Chakravorty, Yutaka Kato, Tsuyoshi Sugiyama, Naoki Koide, Mya Mya Mu, Tomoaki Yoshida, and Takashi Yokochi²

The effect of sodium arsenite (SA) on LPS-induced NO production in RAW 267.4 murine macrophage cells was studied. SA pretreatment of LPS-stimulated RAW cells resulted in a striking reduction in NO production. No significant difference in LPS binding was observed between RAW cells pretreated with SA and control untreated RAW cells, suggesting that SA might impair the intracellular signal pathway for NO production. SA inhibited LPS-induced NF- κ B activation by preventing loss of I κ B- α and - β . Furthermore, SA blocked phosphorylation of extracellular signal-regulated kinase 1/2 (Erk1/2), but not phosphorylation of p38 and c-Jun N-terminal kinase. SA treatment resulted in the disappearance of Raf-1, suggesting that it might cause the inhibition of the Erk1/2 mitogen-activated protein (MAP) kinase pathway. The SA-mediated loss of Raf-1 also abolished LPS-induced NF- κ B activation as well as the Erk1/2 pathway. The dominant negative mutant of MAP kinase kinase 1 inhibited both NO production and NF- κ B activation in LPS-stimulated RAW cells. Taken together, these results indicate that the inhibitory action of SA on NO production in LPS-stimulated macrophages might be due to abrogation of inducible NO synthase induction, and it might be closely related to inactivation of the NF- κ B and Erk1/2 MAP kinase pathways through loss of Raf-1. *The Journal of Immunology*, 2001, 166: 2011–2017.

Nitric oxide (NO) exhibits a wide range of important functions in vivo, acting as a releasing factor mediating vasodilation, as a neuronal messenger molecule, and as a major regulatory molecule and principal cytotoxic mediator of the immune system (1–3). NO is synthesized by constitutively expressed NO synthase (NOS)³ and an inducible isoform of NOS (iNOS) (3, 4). NO production via constitutively expressed NOS and iNOS is regulated in a complicated fashion by various stimuli. Murine macrophages provide the best-studied example of the regulation of NO production (5). NO production is well known to be enhanced by LPS in vitro (6, 7). Augmentation of NO production by LPS is dependent on the expression of iNOS, whose expression is, in turn, mediated by a series of signaling pathway, such as NF- κ B (8–10) and mitogen-activated protein (MAP) kinases (11–14). NO produced in large quantities following induction of iNOS

by LPS in macrophages might play a critical role in endotoxin-induced tissue injury (15, 16). In fact, we have demonstrated that the expression of iNOS and peroxynitrite-induced nitrotyrosine was detected mainly around blood vessels in endotoxin-induced hepatic injury as an experimental endotoxic shock model (17). Sodium arsenite (SA), a standard inducer of heat shock response, is reported to prevent LPS-induced endothelial cell injury (18–20). Recently, we have also reported that SA inhibits LPS-induced bovine endothelial cell injury (21). Therefore, it was of particular interest to determine whether SA affected the production of harmful NO in LPS-stimulated macrophages. In the present study we investigated the effect of SA on LPS-induced NO production in the murine macrophage cell line, RAW 267.4. Herein we describe that SA inhibits LPS-induced NO production by down-regulating extracellular signal-regulated kinase 1/2 (Erk1/2) MAP kinase and NF- κ B pathways through the loss of Raf-1.

Materials and Methods

Materials

LPS from *Escherichia coli* O55:B5 and SA were obtained from Sigma (St. Louis, MO) and Wako Pure Chemicals (Osaka, Japan), respectively. Geldanamycin and pyrrolidine dithiocarbamate (PDTC), purchased from Calbiochem (San Diego, CA), were dissolved to a 1-mM stock solution, which was further diluted in the culture medium for the experiments. The dominant negative mutant of MAP kinase kinase 1 (MEKK1) was supplied by Dr. J. D. Lee (The Scripps Research Institute, La Jolla, CA).

Cell culture

The murine macrophage cell line, RAW 267.4, obtained from the Health Science Resource Bank (Tokyo, Japan), was maintained in RPMI 1640 medium (Sigma) containing 5% heat-inactivated FCS (Life Technologies, Grand Island, NY) at 37°C with 5% CO₂. The cells were washed gently with HBSS (Sigma) and removed from the flasks. The cells were suspended in a 35-mm plastic dish, 96-well plate, or 12-well plate for the experiments.

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³ Abbreviations used in this paper: NOS, NO synthase; SA, sodium arsenite; Erk1/2, extracellular signal-regulated kinase 1/2; JNK/SAPK, c-Jun-N-terminal kinase/stress-activated protein kinase; MAP, mitogen-activated protein; MEKK1, MAP kinase kinase 1; iNOS, inducible isoform of NO synthase; PDTC, pyrrolidine dithiocarbamate; HSP90, 90-kDa heat shock protein.

Pretreatment with SA, geldanamycin, and PDTC

RAW cells were cultured with various concentrations of SA for 90 min, with geldanamycin (3 μM) for 16 h, or with PDTC (100 μM) for 90 min at 37°C. The pretreated cells were washed before use.

Determination of nitrite concentration

NO was measured as its end product, nitrite, using Griess reagent as described previously (22). Culture supernatants (50 μl) were mixed with 100 μl of Griess reagent (1% sulfanilamide, 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride, and 2.5% orthophosphoric acid). After 10 min absorbance at 570 nm was measured in a microplate ELISA reader. The concentration of nitrite in the culture supernatant was determined with reference to a sodium nitrite standard curve. Data represent the mean of triplicate determinations \pm SD. Correction of the NO concentration was not performed because there was no significant difference in the cell number and viability of each experiment.

Laser flow cytometric analysis of CD14 expression, LPS binding, and phagocytosis

RAW cells were pretreated with SA (10 μM) for 90 min. Untreated and SA-pretreated RAW cells were incubated with a 1:200 dilution of FITC-conjugated anti-mouse CD14 Ab (PharMingen, San Diego, CA) or 1 $\mu\text{g}/\text{ml}$ of FITC-conjugated LPS (Sigma) at 4°C for 1 h. Phagocytic activity was estimated by first incubating the cells with 1 $\mu\text{l}/\text{ml}$ of FITC-labeled beads (Polysciences, Warrington, PA) at 37°C for 1 h. The cells were then washed with PBS and suspended in PBS. The fluorescence intensity was analyzed by a laser flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA).

Immunoblotting

Untreated and SA-pretreated RAW cells were seeded in 35-mm plastic dishes (4×10^5 cells/dish) and incubated with LPS for 1 or 8 h. Cells were lysed in the lysis buffer (0.5 M Tris-HCl, 4% SDS, and 2-ME) and boiled for 5 min at 100°C. Aliquots (20 $\mu\text{g}/\text{lane}$) containing equal amounts of protein were electrophoresed under reducing conditions in a 4–20% gradient polyacrylamide gel and transferred to a polyvinylidene difluoride membrane filter. The membranes were treated with 5% BSA for 1 h to block nonspecific binding, rinsed, and incubated with a panel of rabbit polyclonal Abs against 90-kDa heat shock protein (HSP90; StressGene, Victoria, Canada); iNOS (Upstate Biotechnology, Lake Placid, NY); and Raf-1, I κ B- α and - β , Erk1/2, phospho-Erk1/2, p38, phospho-p38, phospho-c-Jun N-terminal kinase/stress-activated protein kinase (phospho-JNK/SAPK), and JNK/SAPK (New England Biolabs, Beverly, MA) for 1 h. The membranes were then treated with a 1:3000 dilution of HRP-conjugated protein G for 1 h. Immune complexes were detected with an enhanced chemiluminescence substrate (New England Nuclear, Boston, MA) and exposed to Kodak XAR x-ray film (Eastman Kodak, Rochester, NY).

Luciferase reporter gene assay for NF- κ B activation

RAW cells ($3 \times 10^5/1$ ml) were plated in 35-mm plastic dishes. On the following day the cells were transfected with 0.5 μg of pNF- κ B-Luc plasmid (a luciferase reporter gene driven by five tandem repeats of NF- κ B, PathDetect System, Stratagene, La Jolla, CA) and 0.5 μg of pCMV- β -gal plasmid (Life Technologies) by the lipofectin method (Life Technologies). The transfected cells were pretreated with SA, geldanamycin, or PDTC for a specified time followed by treatment with LPS (1 $\mu\text{g}/\text{ml}$) for 8 h. The cells were lysed using the lysis reagent from Promega (Madison, WI) before measurement of luciferase activity. The luciferase activity was determined on cell lysates with a luminometer. β -Galactosidase activity was used to normalize transfection efficiencies. All bar diagrams are shown as the mean \pm SD for two experiments in which each transfection was performed in duplicate.

Transfection with antisense oligodeoxynucleotides and the dominant negative MEKK1 mutant

An antisense oligonucleotide to Raf-1 (ATGCATTCTGCCCAAGGA) and a control mismatch antisense oligonucleotide (ATGCAGTCTTCCAC CACGGA) were synthesized by Rikaken (Nagoya, Japan). RAW cells, at \sim 50% confluence, were transfected with either the antisense oligonucleotide to Raf-1 or the control mismatch antisense oligonucleotide (300 nM) using the lipofectin method (Life Technologies) for 8 h. After 8 h, fresh RPMI 1640 containing 10% FCS was added, and the cells were incubated for an additional 24 h before the addition of LPS. The dominant negative MEKK1 mutant was transfected under similar conditions.

Results

SA inhibits NO production in LPS-stimulated RAW cells

The effect of SA pretreatment on LPS-induced NO production in RAW cells was examined (Fig. 1A). RAW cells were pretreated with various concentrations of SA for 90 min, followed by a 24-h incubation with LPS (1 $\mu\text{g}/\text{ml}$). SA pretreatment reduced LPS-induced NO production in a dose-dependent manner, although SA pretreatment at a concentration range of 0.5–100 μM did not affect spontaneous NO production in RAW cells. SA pretreatment also inhibited NO production in response to a higher concentration of LPS (10 $\mu\text{g}/\text{ml}$; data not shown). In addition, there was no significant difference in viability or phagocytic function between SA-pretreated and control untreated RAW cells (data not shown).

The effect of SA on LPS-induced NO production was examined when RAW cells were transiently exposed to SA for various times. The exposure of RAW cells to SA for 5 min resulted in a marked reduction in NO production in response to LPS (1 $\mu\text{g}/\text{ml}$; Fig. 1B),

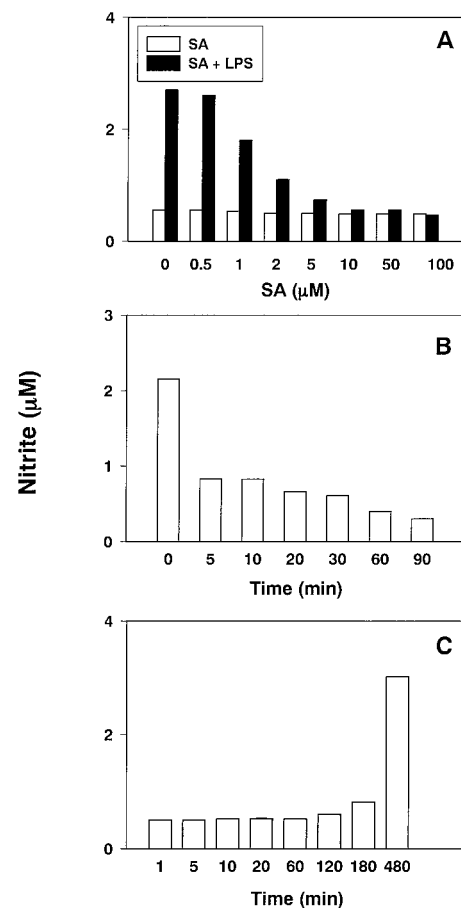


FIGURE 1. The inhibitory action of SA on NO production in LPS-stimulated RAW cells. *A*, The effect of pretreatment with SA on LPS-induced NO production in RAW cells. RAW cells were pretreated with various concentrations of SA for 90 min and washed to remove SA. SA-pretreated RAW cells were incubated with LPS (1 $\mu\text{g}/\text{ml}$) for 24 h. *B*, The effect of exposure time of RAW cells to SA on LPS-induced NO production. RAW cells were exposed to SA (10 μM) for the indicated periods. After removal of SA by washing, SA-pretreated RAW cells were incubated with LPS (1 $\mu\text{g}/\text{ml}$) for 24 h. *C*, The effect of SA post-treatment on NO production in LPS-pretreated RAW cells. RAW cells were pretreated with LPS (1 $\mu\text{g}/\text{ml}$) for the indicated periods. After LPS pretreatment, the cells were washed and cultured with SA (10 μM) for 90 min and subsequently incubated with LPS (1 $\mu\text{g}/\text{ml}$) for 24 h.

suggesting that brief contact of SA was sufficient to inhibit NO production in RAW cells.

The effect of SA post-treatment on LPS-induced NO production was examined (Fig. 1C). RAW cells were incubated with LPS (1 $\mu\text{g/ml}$) for various times, followed by the addition of SA (10 μM) to the cultures. The addition of SA to the cultures of RAW cells pretreated with LPS for 3 h resulted in the inhibition of NO production. However, SA did not inhibit NO production in cells pretreated with LPS (1 $\mu\text{g/ml}$) for 4 h or more.

SA inhibits the expression of iNOS protein in LPS-stimulated RAW cells

Because SA inhibited LPS-induced NO production, the expression of iNOS protein in response to LPS (1 $\mu\text{g/ml}$) was studied in SA-pretreated RAW cells by immunoblotting using an anti-iNOS Ab (Fig. 2). The iNOS protein was readily detected in LPS-stimulated RAW cells. However, pretreatment of RAW cells with SA abrogated the appearance of iNOS in response to LPS. The absence of induction of iNOS protein in SA-pretreated cells was also confirmed by immunofluorescence staining with an anti-iNOS Ab.

SA does not alter the binding of FITC-labeled LPS, the level of cell surface CD14 expression, nor the phagocytic activity in RAW cells

To determine the mechanism of SA inhibition of NO production and iNOS expression in LPS-stimulated RAW cells, we studied the binding of FITC-labeled LPS, the level of cell surface CD14 expression, and the phagocytic activity in control untreated RAW cells and in SA-pretreated RAW cells. Laser flow cytometric analysis demonstrated that there was no significant difference in the binding of FITC-labeled LPS, the level of CD14 expression, or the phagocytic activity between SA-pretreated or control untreated RAW cells (data not shown). This result suggests that SA might affect the intracellular signal transduction of LPS binding to the nucleus or to the effector system in NO production.

SA inhibits the activation of NF- κ B in LPS-stimulated RAW cells

It has been reported that the activation of NF- κ B is involved in LPS-induced NO production in RAW cells (8–10). Therefore, we tested the effect of SA on LPS-induced NF- κ B activation using a luciferase reporter gene assay (Fig. 3A). LPS markedly enhanced reporter gene activity in untreated RAW cells indicating NF- κ B activation. Conversely, pretreatment of cells with SA completely abrogated the enhancement of NF- κ B-dependent reporter gene activity induced by LPS. PDTC, a known inhibitor of NF- κ B, was confirmed to inhibit LPS-induced NF- κ B-dependent reporter gene activity.

The effect of SA on I κ B- α and - β expression was examined to clarify the inhibitory action of SA on LPS-induced NF- κ B activation.

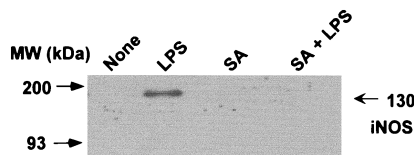


FIGURE 2. The effect of SA on the expression of iNOS protein in LPS-stimulated RAW cells. LPS-induced iNOS expression in SA-pretreated RAW cells was analyzed by immunoblot analysis. SA-pretreated or control untreated cells were incubated with LPS (1 $\mu\text{g/ml}$) for 8 h. The cells were lysed, and the lysates were analyzed by immunoblotting using an anti-iNOS Ab.

(Fig. 3B). The expression of I κ B- α and - β protein was studied in RAW cells following exposure to LPS with or without SA pretreatment by immunoblotting. Although LPS caused marked reduction in the expression of I κ B- α and - β in untreated RAW cells, LPS did not alter the expression of I κ B- α and - β in SA-pretreated cells. This suggests that pretreatment with SA inhibits LPS-induced loss of both I κ B- α and - β .

SA inhibits the phosphorylation of Erk1/2, but not p38 or JNK/SAPK, in LPS-stimulated RAW cells

LPS is known to activate a series of MAP kinases, such as Erk1/2, p38, and JNK/SAPK, in macrophages (11–14). These signal pathways may be involved in LPS-induced NO production in RAW cells. Therefore, the effect of SA on the activation of these MAP kinase pathways was examined by immunoblotting using anti-phospho-MAP kinase Abs (Fig. 4). LPS clearly induced phosphorylated forms of Erk1/2, p38, and JNK/SAPK in untreated RAW cells. However, in SA-pretreated cells, although LPS induced the phosphorylation of p38 and JNK/SAPK, it did not induce the phosphorylation of Erk1/2. SA did not affect the basal level of Erk1/2 expression in RAW cells, but inhibited the phosphorylation of Erk1/2. This suggests that SA may reduce LPS-induced NO production by inhibiting the phosphorylation of the Erk1/2 MAP kinase pathway. It is supported by the finding that PD98059 (20 μM), a MEK inhibitor, inhibits LPS-induced NO production in RAW cells (~50% inhibition).

SA destabilizes Raf-1 and abolishes the phosphorylation of Erk1/2 MAP kinase

We have demonstrated that SA pretreatment abolishes the phosphorylation of Erk1/2 without affecting the basal level of Erk1/2. Based on the fact that Raf-1 plays a critical role in phosphorylation of Erk1/2 via MEK1/2 (23), we examined the effect of SA on the expression level of Raf-1 in RAW cells by immunoblotting (Fig. 5). The level of Raf-1 protein was not significantly altered in RAW

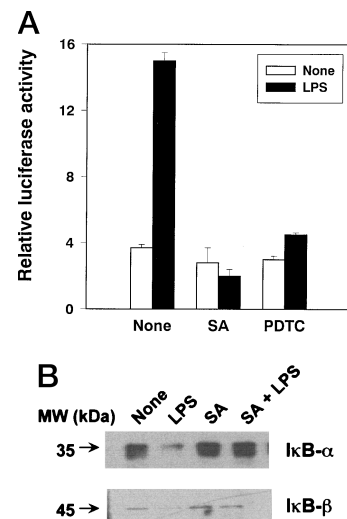


FIGURE 3. The effect of SA on the activation of NF- κ B in LPS-stimulated RAW cells. *A*, The effect of SA on LPS-induced NF- κ B reporter gene activity. RAW cells were cotransfected with NF- κ B-dependent luciferase reporter gene and β -galactosidase gene. After 48 h the cells were pretreated with SA (10 μM) for 90 min or with PDTC (100 μM) for 30 min. The pretreated cells were incubated with LPS (1 $\mu\text{g/ml}$) for 8 h. *B*, The effect of SA on LPS-induced loss of I κ B- α and - β in RAW cells. RAW cells were pretreated with SA (10 μM) for 90 min and incubated with LPS for 1 h. The expression of I κ B- α and - β was determined by immunoblotting using anti-I κ B- α or - β Ab.

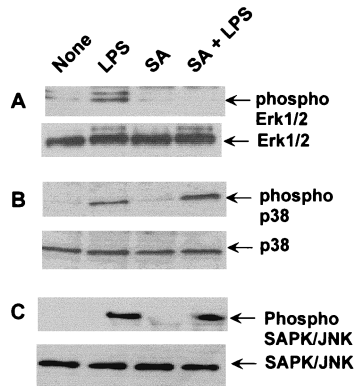


FIGURE 4. The effect of SA on the phosphorylation of Erk1/2, p38, and JNK/SAPK MAP kinases in LPS-stimulated RAW cells. RAW cells were pretreated with SA (10 μ M) for 90 min, followed by treatment with LPS (1 μ g/ml) for 30 min. The cells were lysed, and the lysates were analyzed by immunoblotting using Abs to phospho-Erk1/2 (A), phospho-p38 (B), or phospho-JNK/SAPK (C). The Ab against Erk1/2, p38, or JNK/SAPK was also used.

cells regardless of the presence of LPS. Strikingly, Raf-1 protein was not detected in SA-pretreated RAW cells (Fig. 5A). SA-mediated loss of Raf-1 was detected 5 min after SA treatment (Fig. 5B). Treatment of RAW cells with SA resulted in the disappearance of Raf-1 protein, thereby inhibiting LPS-induced Raf-1-dependent phosphorylation of Erk1/2. Further, we examined whether LPS activated Raf-1 in a Ras-dependent or independent manner. FPTII, a specific inhibitor of Ras, did not inhibit LPS-induced NO production (data not shown), suggesting that LPS activated Raf-1 in a Ras-independent manner.

SA does not affect the expression level of HSP90, which form heteromeric complexes with Raf-1

We have shown that SA treatment of RAW cells results in the disappearance of Raf-1 protein. Three components of the MAP kinase signaling system (Src, Raf, and Mek) are found in association with HSP90, and Raf-1 is stabilized by forming heteromeric complexes with HSP90 (23). Further, NO production is known to be mediated in part through HSP90 signaling (24). Therefore, it was of interest to determine whether SA affects the formation of Raf-1/HSP90 heteromeric complexes (Fig. 6). The effect of SA on the expression of HSP90 in RAW cells was examined by immunoblotting using an anti-HSP90 Ab. The expression of HSP90 was not significantly affected by treatment with LPS or SA alone or by treatment with SA followed by LPS, suggesting no alteration in

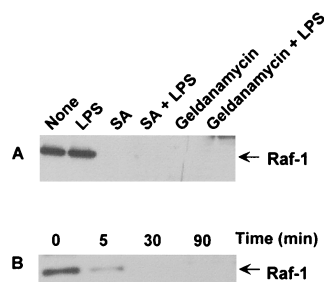


FIGURE 5. The effect of SA on the expression of Raf-1 in LPS-stimulated RAW cells. A, The cells were pretreated with SA (10 μ M) for 90 min or with geldanamycin (3 μ M) for 16 h, followed by treatment with LPS (1 μ g/ml) for 30 min. B, The cells were treated with SA (10 μ M) for various times. The cells were lysed, and the lysates were analyzed by immunoblotting using an anti-Raf-1 Ab.

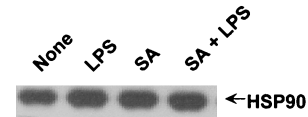


FIGURE 6. The effect of SA on the expression of HSP90 in LPS-stimulated RAW cells. The cells were pretreated with SA (10 μ M) for 90 min and incubated with LPS (1 μ g/ml) for 30 min. The cells were lysed, and the lysates were analyzed by immunoblotting using an anti-HSP90 Ab.

HSP90 expression. To clarify the role of Raf-1/HSP90 heteromeric complexes in LPS-induced NO production in RAW cells, the effect of geldanamycin, which is known to destabilize the preformed heteromeric complexes, on LPS-induced NO production was examined (Fig. 7). The addition of geldanamycin to RAW cells did not affect spontaneous NO production. However, geldanamycin clearly reduced NO production in LPS-stimulated RAW cells, suggesting that the destabilization of Raf-1/HSP90 heteromeric complexes is likely to reduce LPS-induced NO production. In addition, PDTC, an inhibitor of NF- κ B, significantly inhibited LPS-induced NO production, suggesting the participation of NF- κ B.

Raf-1 antisense oligonucleotide inhibits LPS-induced NO production and NF- κ B activation

The effect of the Raf-1 antisense oligonucleotide on LPS-induced NO production was studied (Fig. 8). Introduction of the Raf-1 antisense oligonucleotide into RAW cells significantly suppressed the expression of Raf-1 protein (Fig. 8A). Furthermore, it blocked LPS-induced phosphorylation of Erk1/2 (Fig. 8B). The effect of the Raf-1 antisense oligonucleotide on LPS-induced NO production was examined (Fig. 9). LPS-induced NO production was significantly inhibited in RAW cells transfected with the Raf-1 antisense oligonucleotide (Fig. 9A), but not with the mismatch control. Interestingly, transfection of the Raf-1 antisense oligonucleotide also inhibited NF- κ B activation as well as Erk1/2 phosphorylation in LPS-stimulated RAW cells (Fig. 9B). This strongly suggests that Raf-1 plays a key role in LPS-induced NO production.

Geldanamycin inhibits LPS-induced activation of NF- κ B

We found that Raf-1 antisense oligonucleotide surprisingly inhibited NF- κ B activation in LPS-stimulated RAW cells. Therefore, the effect of geldanamycin, which is known to destabilize the Raf-1/HSP90 heteromeric complexes, on LPS-induced NF- κ B activation was examined using the luciferase reporter gene assay (Fig.

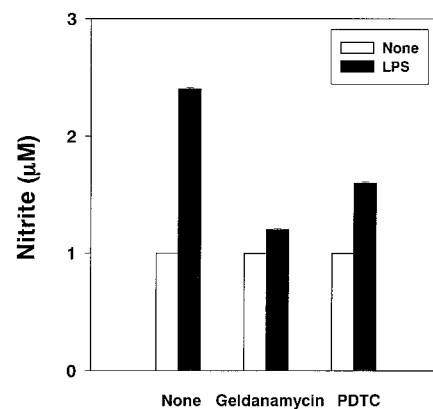


FIGURE 7. The effect of geldanamycin on LPS-induced NO production in RAW cells. The cells were pretreated with geldanamycin (3 μ M) for 16 h or with PDTC (100 μ M) for 30 min and incubated with LPS (1 μ g/ml) for 24 h.

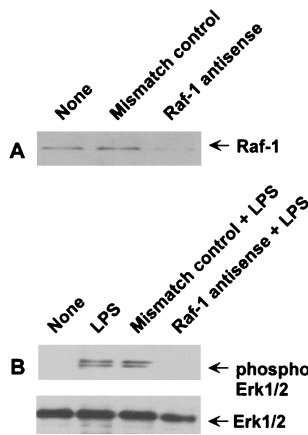


FIGURE 8. The effect of the Raf-1 antisense oligonucleotide on Raf-1 expression (A) and phosphorylation of Erk1/2 (B) in LPS-stimulated RAW cells. A, RAW cells were treated with the Raf-1 antisense oligonucleotide or mismatch control for 48 h. The cells were lysed, and the lysates were analyzed by immunoblotting using an anti-Raf-1 Ab. B, RAW cells were pretreated with the Raf-1 antisense oligonucleotide or a mismatch control for 48 h and incubated with LPS (1 $\mu\text{g}/\text{ml}$) for 30 min. The cells were lysed, and the lysates were analyzed by immunoblotting using anti-phospho-Erk1/2 or -Erk1/2 Ab.

10). Geldanamycin blocked the LPS-induced enhancement of NF- κB -dependent reporter gene activity. Therefore, it appears that destabilization of Raf-1/HSP90 by geldanamycin causes the inhibition of LPS-induced NF- κB activation.

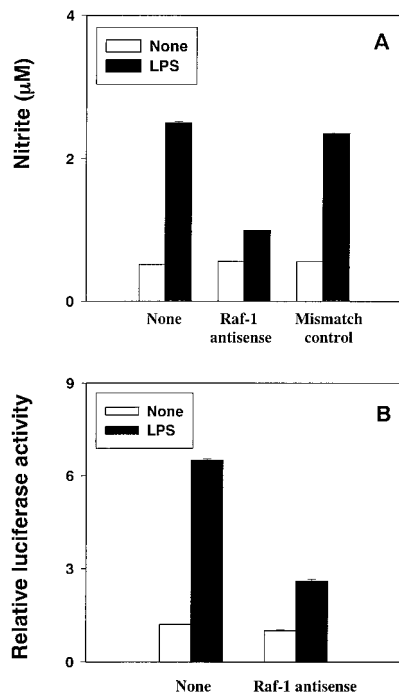


FIGURE 9. The effect of the Raf-1 antisense oligonucleotide on NO production (A) and NF- κB activation (B) in LPS-stimulated RAW cells. A, RAW cells were pretreated with the Raf-1 antisense oligonucleotide or mismatch control for 48 h and incubated with LPS (1 $\mu\text{g}/\text{ml}$) for 18 h. B, RAW cells were transfected with the Raf-1 antisense oligonucleotide or mismatch control for 48 h and further cotransfected with NF- κB dependent luciferase reporter gene along with β -galactosidase for 48 h. The cells were incubated with LPS (1 $\mu\text{g}/\text{ml}$) for 8 h.

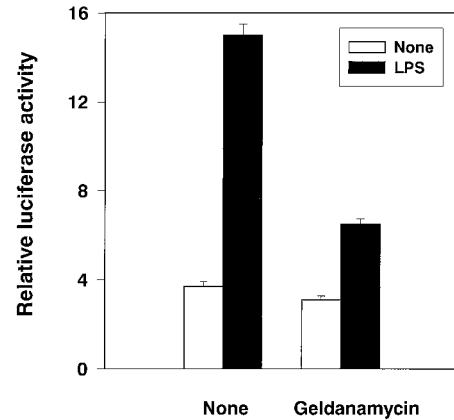


FIGURE 10. The effect of geldanamycin on NF- κB activation in LPS-stimulated RAW cells. RAW cells were cotransfected with NF- κB -dependent luciferase reporter gene and β -galactosidase. After 48 h the cells were pretreated with geldanamycin (3 μM) for 16 h. The pretreated cells were incubated with LPS (1 $\mu\text{g}/\text{ml}$) for 8 h.

Effect of the dominant negative mutant of MEKK1 on NF- κB activation and NO production in LPS-stimulated RAW cells

Raf-1 has been recently reported to activate MEKK1 (25) and result in NF- κB translocation (26). Thus, the SA-mediated loss of Raf-1 protein may abolish Raf-1-mediated MEKK1 activation, leading to a loss of NF- κB induction. The effect of a dominant negative MEKK1 mutant on NO production and NF- κB activation in LPS-stimulated RAW cells was studied (Fig. 11). Transfection of RAW cells with the dominant negative MEKK1 mutant did not affect the constitutive expression of Raf-1 (data not shown), suggesting that Raf-1 is the upstream activator of MEKK1. As shown

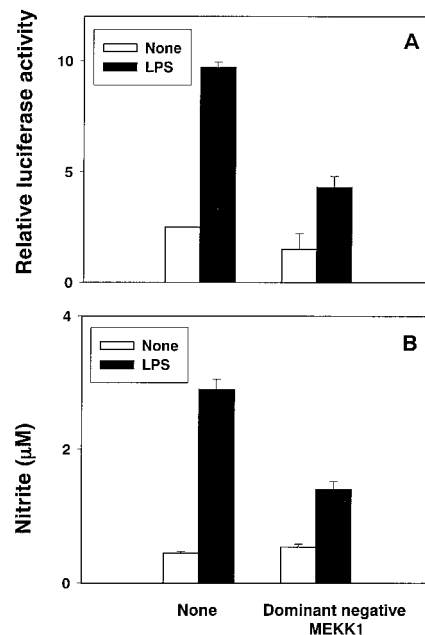


FIGURE 11. The effect of the dominant negative mutant of MEKK1 on NF- κB activation (A) and NO production (B) in LPS-stimulated RAW cells. A, RAW cells were cotransfected with the dominant negative mutant of MEKK1, NF- κB -dependent luciferase reporter gene, and β -galactosidase gene for 48 h. The transfected cells were incubated with LPS (1 $\mu\text{g}/\text{ml}$) for 8 h. B, RAW cells were transfected with the dominant negative MEKK1 for 48 h. The transfected cells were incubated with LPS (1 $\mu\text{g}/\text{ml}$) for 18 h.

in Fig. 11A, transfection of the dominant negative MEKK1 mutant resulted in the striking inhibition of LPS-induced NF- κ B activation (50% reduction). LPS-induced NO production was also inhibited in cells transfected with the dominant negative MEKK1 mutant (Fig. 11B). The inhibition by the dominant negative MEKK1 mutant was less than that observed by treatment with SA.

Discussion

In the present study we demonstrate that SA inhibits the expression of iNOS and causes reduced NO production in LPS-stimulated RAW murine macrophage cells. The inhibition of iNOS expression by SA is not due to altered LPS binding or cell damage. Rather, the inhibition appears to be mediated through the impairment of specific intracellular signals. In fact, SA down-regulates NF- κ B and Erk1/2 MAP kinase pathways, but not p38 or JNK MAP kinase pathways. In particular, the inhibition of NF- κ B activation by SA is important for the suppression of LPS-induced iNOS expression, because the activation of NF- κ B plays a critical role in the expression of iNOS in LPS-stimulated macrophages (8–10). Furthermore, we demonstrate that SA down-regulates the Erk1/2 MAP kinase signal pathway in which Raf-1 activates MEK1/2, which, in turn, activates the Erk1/2 kinases. There are several reports that iNOS expression in LPS-stimulated macrophages is mediated by the Erk1/2 MAP kinase pathway (11–13). We also demonstrate the inhibition of LPS-induced NO production by a MEK inhibitor, PD98059. This strongly suggests that the inhibitory action of SA on LPS-induced NO production is due to abrogation of iNOS induction through the down-regulation of NF- κ B and/or Erk1/2 MAP kinase signal pathways.

Raf-1 is a component of the Erk1/2 MAP kinase signal system (27), suggesting that loss of Raf-1 causes the impairment of Erk signaling pathway. However, the loss of Raf-1 by antisense oligonucleotide, geldanamycin, and SA also down-regulated LPS-induced NF- κ B activation. How could the Raf-1 molecule lead to the activation of NF- κ B? Recently, Bauman et al. (26) have reported that Raf-1 mediates the activation of NF- κ B through MEKK1. This evidence may elucidate our finding that SA-mediated Raf-1 loss resulted in the down-regulation of NF- κ B activation and inhibited the expression of iNOS in LPS-stimulated RAW cells. In fact, we demonstrate that the dominant negative mutant of MEKK1 down-regulates LPS-induced NF- κ B activation and NO production without affecting Raf-1 expression. Taken together, these results show that SA causes the loss of Raf-1, resulting in the down-regulation of MEKK1 activity and the subsequent inhibition of NF- κ B activation. Thus, the loss of Raf-1 reduced NO production in LPS-stimulated RAW cells through the inhibition of NF- κ B and Erk activity, demonstrating that Raf-1 plays a critical role in LPS-induced NO production in macrophages. In addition, SA is reported to inhibit NF- κ B-mediated gene transcription by blocking I κ B kinase activity and I κ B- α phosphorylation and degradation (28).

SA induced the disappearance of Raf-1, which is the immediate upstream activator of MEK1/2 and the upstream activator of Erk1/2 MAP kinase signal pathway. Therefore, it is reasonable that SA abrogates the Erk1/2 MAP kinase pathway through the loss of Raf-1 in LPS-stimulated RAW cells. Of particular interest are a series of recent reports implicating HSP90 and its associated proteins in signal transduction of macrophage activation (24, 25). Raf-1 protein exists in native complexes with HSP90 that can be formed in vitro by reticulocyte lysate, and its catalytic domain is sufficient for HSP90 binding (29, 30). Based on the fact that geldanamycin, which binds to HSP90 and leads to a block in Raf-1/HSP90 heteromeric complex assembly (31, 32), significantly inhibits LPS-induced NO production in RAW cells, the Raf-1/HSP90 heteromeric complexes are likely to participate in iNOS

induction in LPS-stimulated RAW cells. It was shown that SA inhibits the Erk1/2 MAP kinase pathway by the loss of Raf-1 without affecting the expression of HSP90. The critical role of Raf-1 is also supported by the results showing that LPS-induced NO production was significantly inhibited in RAW cells transfected with the Raf-1 antisense oligonucleotide. Recently, Byrd et al. (24) reported that HSP90 can bind LPS and mediates macrophage activation by LPS. However, the exact action of LPS on Raf-1/HSP90 complex has not yet been clarified.

A series of signaling pathways, including NF- κ B, Erk1/2 kinase, p38 MAP kinase, and JNK/SAPK MAP kinase pathways, is involved in the induction of iNOS in LPS-stimulated macrophages. The present study demonstrates an intimate cross-talk between the NF- κ B and Erk1/2 pathways in iNOS expression. This is supported by several reports that NF- κ B transcriptional activity is controlled by Erk1/2 MAP kinase (33–36). In addition, the signaling pathways with p38 MAP kinase and SAPK/JNK MAP kinase are reported to be involved in LPS-induced iNOS expression (13, 37). Although SA did not inhibit the phosphorylation of p38 and JNK/SAPK MAP kinases, SA completely inhibited NO production in LPS-stimulated RAW cells. It did not exclude the participation of p38 and JNK/SAPK in LPS-induced NO production. Thus, NO production in LPS-stimulated macrophages is regulated through a complex signaling system via the NF- κ B and MAP kinase pathways.

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