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Oxidant Stress Incites Spreading of Macrophages via Extracellular Signal-Regulated Kinases and p38 Mitogen-Activated Protein Kinase

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Cultured macrophages exhibit spreading in response to external stimuli. It is relevant to in vivo morphologic changes of macrophages during extravasation, migration, and differentiation. The present study was performed to elucidate molecular mechanisms that regulate spreading of macrophages. Redox is a crucial factor that modulates a wide range of cell function. We found that macrophages undergo spreading in response to oxidant stress caused by hydrogen peroxide or an oxidant generating agent menadione. To identify signaling pathways involved, a role of mitogen-activated protein (MAP) kinases was investigated. Western blot analysis showed that treatment of macrophages with menadione rapidly induced phosphorylation of extracellular signal-regulated kinases (ERK1, ERK2) and p38 MAP kinase, but not c-Jun N-terminal kinase (JNK). Pharmacologic inhibition of either ERK or p38 activation blunted the macrophage spreading. Similarly, transfection with dominant-negative mutants of ERKs or a mutant p38 significantly suppressed the oxidant-triggered spreading. ERKs and p38 are known to activate serum response element (SRE) via phosphorylation of the ternary complex factor Elk-1. To further identify downstream events, we focused on a role of SRE. Stimulation of macrophages with menadione induced activation of SRE. Intervention in the SRE activation by a dominant-negative mutant of Elk-1 inhibited the menadione-induced spreading. These results suggest that oxygen radical metabolites, the well-known mediators for tissue injury, incite spreading of macrophages via the MAP kinase-SRE signaling pathways. The Journal of Immunology, 1998, 161: 3569–3574.

Macrophages and pharmacologic manipulation

The normal alveolar rat macrophage NR8383 (12) was purchased from American Type Culture Collection (ATCC, Manassas, VA). The cells were maintained in DMEM/Ham’s F-12 (DMEM-F12; Life Technologies, Gaithersburg, MD) supplemented with 100 U/ml penicillin G, 100 μg/ml of streptomycin, 0.25 μg/ml amphotericin B, and 10 to 15% FCS.

For induction of spreading, NR8383 macrophages seeded in 24-well tissue culture plates (Becton Dickinson, Lincoln Park, NJ) were stimulated with menadione (2-methyl-1,4-naphthoquinone; 25 to 100 μM; Sigma, St. Louis, MO) or 300 μM hydrogen peroxide (Sigma). Menadione is a quinone compound that generates superoxide (O$_2$) via one-electron-transfer reactions and produces H$_2$O$_2$ and hydroxyl radical (OH$^-$) (13). Menadione, 50 to 100 μM, was generally used as an inducer of spreading. Morphologic examination was performed after 1 h using phase-contrast microscopy. The
percentage of spreading cells was counted in each well, and the mean value of four wells was used to compare data in different groups. More than 60 cells per well were randomly examined for the evaluation. In some experiments, macrophages were pretreated with antioxidant N-acetyl-l-cysteine (10 mM; Sigma) for 2 h and then stimulated by menadione.

To examine roles of ERKs and p38 MAP kinase in the oxidant-triggered spreading, NR8383 macrophages were pretreated with PD098059 (10–75 μM; a gift from Dr. A. R. Saltiel, Parke-Davis Pharmaceutical Research, Ann Arbor, MI) (14) or SB203580 (10 μM; Calbiochem-Novabiochem, Nottingham, U.K.) for 2 h and then stimulated by menadione. All experiments were performed in quadruplicate.

Transfection

The role of ERKs in the oxidant-triggered cell spreading was investigated by transient transfection. Using a modified calcium phosphate coprecipitation method (15), NR8383 macrophages were transfected with pCI-Jnk1 (a gift from Promega, Madison, WI) together with expressing vector pCEP4Erk1 + pCEP4Erk2 or pCEP4Erk1(K171R) + pCEP4Erk2(K52R), as described below. pCI-Jnk1 introduces a β-galactosidase gene under the control of the immediate-early enhancer/promoter of human cytomegalovirus, pCEP4Erk1 and pCEP4Erk2 code for wt ERK1 and ERK2, pCEP4Erk1(K171R) and pCEP4Erk2(K52R) encode dominant-negative mutants of ERK1 and ERK2, respectively. When overexpressed, these mutants effectively suppress the function of endogenous ERK1 and ERK2 (16). NR8383 cells (2 × 10⁵) were suspended in 1 ml of calcium phosphate-DNA complex containing 2 μg pCI-Jnk1 + 3 μg pCEP4Erk1 + 3 μg pCEP4Erk2, or 2 μg pCI-Jnk1 + 3 μg pCEP4Erk1(K171R) + 3 μg pCEP4Erk2(K52R). After incubation for 20 min, growth medium (10–15% FCS/DMEM-F12; 9 ml) was added, incubated for additional 2 h, and treated with chloroquine at a concentration of 50 μg/ml. After 2 h incubation, cells were washed and exposed to 1% glutaraldehyde in PBS for 90 s. Then, the cells were washed and seeded in 12-well plates. After incubation for 4 h, cells were reseeded in 6-well plates or 10-cm tissue culture plates, stimulated by 100 μM menadione for 1 h, and subjected to 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) assay. To test contribution of p38 MAP kinase, NR8383 macrophages were transfected with pCI-Jnk1 (2 μg) together with empty vector pcDNA3 (6 μg) or pcDNA-p38(TY) (6 μg) encoding a dominant negative mutant of p38 (17).

To examine a role of SRE in the oxidant-triggered spreading of macrophages, a dominant-negative mutant of Elk-1 was used (18). Elk-1-interacting complexes to SRE leads to induction of target genes. If the dominant-interfering form of Elk-1 is overexpressed, activity of SRE is suppressed (18). NR8383 macrophages were transiently transfected with pCI-Jnk1 (3 μg) together with an empty plasmid pcDNA3 (6 μg) or pDEN-Elk (6 μg) encoding a mutated Elk-1 with deletion of the carboxyl-terminal activation domain. After 48 h, cells were exposed to menadione and subjected to X-gal assay. All transfection experiments were performed in quadruplicate.

X-gal assay

X-gal assay was performed as described before (19). In brief, cells were fixed in 0.5% glutaraldehyde, 2 mM MgCl₂, and 1.25 mM EGTA in PBS for 10 min at room temperature and incubated at 37°C for 2 h in X-gal solution containing 1 mg/ml X-gal (Sigma), 20 mM K₃Fe(CN)₆, 20 mM K₄Fe(CN)₆·3H₂O, 2 mM MgCl₂, 0.01% sodium deoxycholate and 0.02% Nonidet P-40 in PBS (pH 7.4). Percentage of spreading blue cells against total number of blue cells was calculated in each well, and the mean value of four wells was used to compare data in different groups.

Western blot analysis

Phosphorylated forms of ERKs and p38 were detected by Western blot analysis. NR8383 macrophages (1–2 × 10⁶/well) cultured in six-well plates were stimulated by 75 μM menadione for 15 to 60 min. After the exposure, cells were rinsed by PBS, lysed with 300 μl of sample buffer (4% SDS, 10% glycerol, 0.006% bromophenol blue, and 2% β-mercaptoethanol in 250 mM Tris-HCl, pH 6.8) and boiled for 5 min. Samples were passed several times through 23-gauge needles. After centrifugation, supernatants were electrophoresed in 10% acrylamide gels and transferred onto nitrocellulose membranes. Analyses were performed using PhosphoPlus MAPK Phospho Ab Kit and PhosphoPlus p38 MAP Kinase Ab Kit (New England Biolabs, Hitchin, U.K.) following protocols provided by the manufacturer.

Activity of JNK was evaluated by phosphorylation of c-Jun, using SAPK/JNK Assay Kit (New England Biolabs). In brief, after exposure to menadione, cells were lysed with 300 μl of lysis buffer and passed several times through needles. After centrifugation, each supernatant containing 50 μg of total protein was incubated with 1 μg of c-Jun fusion protein beads at 4°C overnight. After centrifugation, the pellets were washed, suspended in 50 μl of kinase buffer supplemented with 100 μM ATP, and incubated for 30 min at 30°C. Then, 50 μl of sample buffer was added to each, boiled for 5 min, and centrifuged. Supernatants were then subjected to electrophoresis and immunoblot analysis following the protocol provided by the kit.

Northern blot analysis

NR8383 macrophages were exposed to 50 μM menadione for 0.5, 1, and 2 h. Total RNA was extracted by a single-step method (20) and subjected to Northern blot analysis, as described before (21). In brief, RNA samples were electrophoresed on 1.2% agarose gels containing 10% formaldehyde and transferred onto nitrocellulose membranes. A mouse erb-1 cDNA (22), a human c-fos cDNA (23), and a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were labeled with [³²P]dCTP using the random priming method. The membranes were hybridized with probes at 65°C overnight in a solution containing 4X SSC (600 mM sodium chloride, 60 mM sodium citrate), 5X Denhardt’s solution, 10% dextran sulfate, 50 μg/ml herring sperm DNA, and 50 μg/ml poly(A), washed at 50°C, and exposed to Kodak XAR films at −80°C.

Statistical analysis

Data were expressed as means ± SE. Statistical analysis was performed using the nonparametric Mann-Whitney U test to compare data in different groups. We used a value of p < 0.05 to indicate a statistically significant difference.

Results

Induction of macrophage spreading by oxidant stress

To investigate the effect of oxidants on macrophage spreading, NR8383 cells were plated on tissue culture plastic and incubated in the absence or presence of menadione, which generates reactive oxygen intermediates. Within 1 h, the majority of cells adhered on plastic and showed round appearance, with bright cytoplasm and clear margin (Fig. 1A, left). After exposure to menadione, macrophages altered their shape within 30 min and exhibited the spreading form” characterized by a flat and ameboid shape and dark cytoplasm with vacuolation (1, 24) (Fig. 1A, right). After 1 h, approximately 60% of stimulated macrophages showed spreading. The effect of menadione was dose dependent; i.e., percentages of spreading cells were 9.6% by 25 μM, 25.5% by 50 μM, 49.9% by 75 μM, and 55.5% by 100 μM, compared with untreated control (4.8%) (Fig. 1B). This effect was completely abrogated in the presence of antioxidant N-acetyl-l-cysteine (Fig. 1C). The stimulatory effect of oxidant stress on the cell spreading was further confirmed using H₂O₂. As shown in Figure 1D, 300 μM H₂O₂ induced spreading of macrophages (53.5 ± 4.1%, mean ± SE, p < 0.05) similarly to the effect of menadione.

Involvement of ERKs

To identify signaling pathways involved in the oxidant-initiated macrophage spreading, a role of ERKs was investigated. NR8383 macrophages were treated with menadione for 15 min, and phosphorylation of ERK1 and ERK2 was examined using phospho-specific Abs. Western blot analysis showed that stimulation of macrophages with menadione rapidly induced phosphorylation of ERK1 and ERK2 (Fig. 2A).

To examine whether the activation of ERKs is essential for the oxidant-induced cell spreading, NR8383 macrophages were pre-treated with serial concentrations of the MAP kinase kinase inhibitor PD098059 and then stimulated by menadione. Microscopic analysis showed that PD098059 inhibited cell spreading in a dose-dependent manner (Fig. 2B). Significant suppression was observed at concentrations higher than 50 μM. Compared with control (42.7 ± 2.8%), the percentages of spreading cells were reduced to 28.6 ± 1.6% (50 μM) and 26.0 ± 3.0% (75 μM).

The crucial role of ERKs was further confirmed by a transient transfection assay. NR8383 cells were transfected with a reporter
plasmid pCI-bGal together with pCEP4Erk11pCEP4Erk2 encoding wt ERKs or pCEP4Erk1(K71R)pCEP4Erk2(K52R) coding for dominant-interfering forms of ERKs. After incubation for 48 h, cells were stimulated with menadione and subjected to

**FIGURE 1.** Induction of macrophage spreading by oxidant stress. A, Morphologic examination of menadione-induced macrophage spreading. NR8383 rat macrophages were plated on tissue culture plastic and incubated for 1 h in the absence (left) or presence (right) of 75 μM menadione, which generates reactive oxygen intermediates. Phase-contrast microscopy. B, Dose-dependent effect of menadione. NR8383 cells were exposed to menadione at concentrations ranging from 25 μM to 100 μM, and percentages of spreading cells were evaluated by phase-contrast microscopy. C, Inhibition of the menadione-induced spreading by antioxidant N-acetyl-L-cysteine (NAC). NR8383 macrophages were pretreated with or without 10 mM NAC for 2 h and stimulated by menadione. Assays were performed in quadruplicate, and data were presented as means ± SE. An asterisk indicates a statistically significant difference (p < 0.05) compared with the cells treated with menadione alone. D, Induction of macrophage spreading by hydrogen peroxide (H2O2). NR8383 cells were stimulated by 300 μM H2O2 for 1 h, and percentages of spreading cells were evaluated. Assays were performed in quadruplicate. An asterisk indicates a statistically significant difference (p < 0.05) compared with untreated control.

**FIGURE 2.** Involvement of extracellular signal-regulated kinases (ERKs) in the oxidant-triggered macrophage spreading. A, Activation of ERK1 and ERK2 in menadione-treated macrophages. NR8383 macrophages were treated with 75 μM menadione for 15 min, and phosphorylation of ERK1 and ERK2 was examined by Western blot analysis. As loading controls, the amount of ERK1 and ERK2 proteins are shown on the bottom. B, Dose-dependent inhibition of oxidant-triggered spreading by the mitogen-activated protein kinase kinase inhibitor PD098059. NR8383 macrophages were pretreated with serial concentrations of PD098059 (10–75 μM), stimulated by 50 μM menadione for 1 h, and percentages of spreading cells were evaluated. Assays were performed in quadruplicate. Asterisks indicate statistically significant differences (p < 0.05) compared with the control without PD098059. C, Inhibition of oxidant-triggered spreading by dominant-negative mutants of ERKs. NR8383 cells were transiently transfected with a reporter plasmid pCI-bGal together with pCEP4Erk1 + pCEP4Erk2 encoding wild-type ERKs (wt ERKs) or pCEP4Erk1(K71R) + pCEP4Erk2(K52R) coding for dominant-interfering forms of ERKs (ΔERKs), as described in Materials and Methods. After incubation for 48 h, cells were stimulated with menadione for 1 h and subjected to X-gal assay. Percentages of spreading cells were evaluated using X-gal-positive cells. Assays were performed in quadruplicate. Percentage of spreading blue cells against total number of blue cells was calculated in each well, and the mean value was used to compare data in different groups. The result (mean ± SE) is shown as a relative percentage against the value of control (100%). An asterisk indicates a statistically significant difference (p < 0.05) compared with the cells transfected with wt ERKs.
**FIGURE 3.** Involvement of p38 MAP kinase, but not JNK, in the oxidant-triggered macrophage spreading. A, Activation of p38 in menadione-treated macrophages. NR8383 macrophages were stimulated by 75 μM menadione, and, after 15 to 60 min, phosphorylation of p38 was examined by Western blot analysis. As a loading control, the amount of c-Jun protein is shown on the bottom. B, Activity of JNK in menadione-treated macrophages. NR8383 cells were stimulated by menadione, and phosphorylation of c-Jun was examined by Western blot analysis. As a loading control, the amount of c-Jun protein is shown on the bottom. C, Inhibition of oxidant-triggered spreading by the p38 inhibitor SB203580. NR8383 macrophages were pretreated with SB203580 (10 μM) together with or without PD098059 (50 μM) for 2 h and stimulated by menadione for 1 h. Assays were performed in quadruplicate. An asterisk indicates a statistically significant difference (p < 0.05) compared with the control treated with menadione alone. A double asterisk indicates a significant difference compared with the cells treated with menadione and SB203580. D, Inhibition of oxidant-triggered spreading X-gal assay. Percentages of spreading cells were evaluated using X-gal-positive cells. As shown in Figure 2C, transfection with dominant-negative mutants significantly inhibited macrophage spreading. Compared with control (100%), the relative percentage of spreading cells was reduced to 60.8 ± 9.4% (p < 0.05) by the inhibition of ERKs.

**Involvement of p38 MAP kinase and JNK**

Pharmacologic and genetic inactivation of ERKs only partially inhibited the oxidant-induced macrophage spreading. To further identify molecules that cooperate with ERKs, involvement of p38 MAP kinase and JNK was tested. NR8383 macrophages were treated with menadione for 15 to 60 min, and phosphorylation of p38 and c-Jun was examined. Western blot analysis showed that treatment of macrophages with menadione rapidly induced phosphorylation of p38, with a peak at 30 min (Fig. 3A). In contrast, JNK was constitutively active in NR8383 cells, and its activation state was not affected in response to the oxidant stress (Fig. 3B).

To examine whether the activation of p38 is required for the oxidant-induced cell spreading, NR8383 cells were pretreated with a pharmacologic inhibitor of p38, SB203580, and then stimulated by menadione. Morphologic analysis showed that SB203580 partially inhibited cell spreading (Fig. 3C). Compared with control (40.9 ± 3.3%), the percentage of spreading cells was reduced to 24.6 ± 2.1%. Pretreatment of the cells with SB203580 together with PD098059 led to dramatic suppression of the macrophage spreading (6.8 ± 0.9%).

The role of p38 was further confirmed by a transient transfection assay. NR8383 cells were transfected with a reporter plasmid pCIBgal together with an empty plasmid pcDNA3 or pcDNA3-p38(TY) that encodes a dominant-negative mutant of p38. After incubation for 48 h, cells were stimulated with menadione and subjected to X-gal assay. As shown in Figure 3D, transfection with p38(TY) significantly inhibited macrophage spreading. Compared with control (100%), the relative percentage of spreading cells was reduced to 57.9 ± 7.5% by the inhibition of p38.

**Involvement of SRE**

ERKs and p38 are known to activate SRE via phosphorylation of a ternary complex factor Elk-1 (9). To further identify molecular events downstream of the MAP kinase activation, we examined the involvement of SRE. Expression of egr-1 and c-fos are regulated by SREs located in their 5'-flanking regions (8, 25). Using these genes as indicators, activity of SRE was tested by Northern blot analysis. After stimulation of macrophages with menadione, expression of both egr-1 and c-fos was rapidly induced within 30 min and peaked at 1 h (Fig. 4A). To examine whether activation of SRE is required for the oxidant-triggered spreading, a dominant-negative mutant of Elk-1 was used. Elk-1 forms complexes with SRF, and binding of Elk-1-SRF complexes to SRE leads to induction of target genes. If the dominant-interfering form of Elk-1 is overexpressed, activity of SRE is abrogated (18). NR8383 macrophages were transiently transfected with pCI-βGal together with an empty plasmid pCMV5 or pDN-Elk. After 48 h, cells were exposed to menadione, and X-gal assay was performed. As shown in Figure 4B, by a dominant-negative mutant of p38. NR8383 cells were transiently transfected with a reporter plasmid pCIBgal together with an empty plasmid pcDNA3 or pcDNA3-p38(TY) that encodes a dominant-negative mutant of p38. After incubation for 48 h, cells were stimulated with menadione (100 μM) for 1 h and subjected to X-gal assay. An asterisk indicates a statistically significant difference (p < 0.05).
of receptors for leukocyte adhesion, leading to accumulation of circulating monocytes at affected sites (30, 31). Our current results, together with the previous reports, suggest potential roles of oxygen radical intermediates for local macrophage function. At the site of inflammation, oxidant stress may attract monocytes and facilitate their extravasation, migration, and differentiation to tissue macrophages. Of note, in the pathologic milieu, the monocyte/macrophage is an important source of local oxidant production. Oxygen radical intermediates produced by local macrophages may further facilitate accumulation and activation of macrophages.

Intracellular signaling pathways involved in macrophage spreading are poorly understood. Previous reports suggested possible roles of certain signaling molecules, including protein kinase C, cytosolic free calcium, phospholipase A2, and arachidonic acid (2, 3, 32, 33). To date, however, successive signaling cascades involved in the macrophage spreading are not identified. In the present investigation, we highlighted the role of the MAP kinase-SRE pathways. ERKs, JNK, and p38 MAP kinase are known to be redox sensitive and activated by oxidants in certain cell types (10, 34). SRE is also regarded as an oxidant-responsive regulatory element (11, 35). Consistent with these, our results showed that menadione phosphorylated ERKs and p38, leading to activation of SRE. Inhibition of either step partially suppressed the oxidant-mediated spreading of macrophages. When both pathways were concomitantly blocked, dramatic suppression of macrophage spreading was observed. Similar, substantial suppression was also achieved by inactivation of SRE. These results indicate that, in response to oxidant stress, the ERK pathway and the p38 pathway cooperatively incite macrophage spreading via activation of SRE.

Rho family members of GTP-binding proteins (Rho, Rac, and Cdc42) play a role in alteration in cytoskeleton organization, motility, and cell shape (36, 37). It has been reported that these GTP-binding proteins can be activated in response to oxidant stress (38) and participate in phorbol ester-induced spreading of macrophages (39). The activation of the Rho family molecules leads to phosphorylation of JNK (40), which may phosphorylate Elk-1 and activate SRE (41). Although, in NR8383 cells, the activity of JNK was not affected by the oxidant stress, the Rho-JNK-SRE pathway might, in part, participate in the oxidant-induced spreading in other cells of monocyte/macrophage lineage.

Molecular events downstream of SRE are currently undetermined. Since the spreading of macrophages is rapid, some immediate-early genes may be involved. A previous report has shown that expression of egr-1 is essential for phorbol ester-induced spreading of myeloid leukemia cells and normal myeloblasts (7). As is known, the expression of egr-1 is induced by oxidants and regulated by SREs in its 5′-flanking region (8, 42). We observed that menadione transiently induced the expression of egr-1 in macrophages preceding their spreading. However, our preliminary results showed that 1) inhibition of egr-1 expression by antisense oligodeoxynucleotides did not inhibit the oxidant-induced spreading of macrophages, and 2) transient transfection with a full-length egr-1 cDNA failed to induce macrophage spreading. Similarly, transfection with a c-fos cDNA, another oxidant inducible immediate-early gene controlled by SRE (25), did not result in macrophage spreading (our unpublished observation). These data imply a possibility that downstream molecule(s) other than egr-1 and c-fos may be involved in the oxidant-initiated macrophage spreading.
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