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Lipopolysaccharide Induces Matrix Metalloproteinase-9 Expression via a Mitochondrial Reactive Oxygen Species-p38 Kinase-Activator Protein-1 Pathway in Raw 264.7 Cells

Chang-Hoon Woo, Jae-Hyang Lim,2 and Jae-Hong Kim3

We have identified a novel signaling pathway that leads to expression of matrix metalloproteinase-9 (MMP-9) in murine macrophages in response to the bacterial endotoxin, LPS. We showed that p38 kinase was essential for this induction and observed that LPS-induced MMP-9 expression was sensitive to rottlerin, a putative protein kinase C (PKC) inhibitor. However, neither infection with a retrovirus expressing a dominant negative mutant of PKCα nor down-regulation of PKCα by prolonged PMA treatment affected MMP-9 expression, thus excluding involvement of PKCα. Interestingly, LPS-induced MMP-9 expression and p38 kinase phosphorylation were shown to be suppressed by the antioxidant diphenyleneiodonium chloride (DPI) (15), and a recent report suggests that DPI is also a potent inhibitor of mitochondrial ROS.

MMPs are not only produced by structural cells such as fibroblasts and endothelial and epithelial cells, but also by inflammatory cells, including macrophages, lymphocytes, neutrophils, and eosinophils (6). Monocytes/macrophages are prominent at chronic inflammatory sites such as areas of arthritis, atherosclerosis, and periodontal disease, in which degradation of the connective tissue is believed to contribute substantially to the pathology of the disease. Stimulation of monocytes with LPS, a surface component of Gram-negative bacteria, induces a number of MMPs, including two prominent monocyte MMPs: MMP-1, an interstitial collagenase, and MMP-9 (gelatinase B; 92-kDa type IV collagenase). MMP-9 degrades laminin and type IV collagen, two components of the basement membrane. Thus, it is involved in the connective tissue loss associated with such chronic inflammatory diseases as asthma, pulmonary fibrosis, and chronic obstructive pulmonary disease, as well as with cancer progression. For instance, the level of MMP-9 is elevated in the bronchoalveolar lavage of asthmatic patients and gives rise to tissue remodeling of the airways (7).

The activity of MMPs is tightly regulated in terms of gene expression, spatial localization, and proenzyme activation; it is also subject to naturally occurring tissue MMP inhibitors (8). MMP-9 expression appears to be regulated by a number of different signaling pathways in different cell types. Thus, protein kinases, MAPKs, transcription factors such as NF-κB, and AP-1 have all been reported to be involved in the induction of MMPs (9, 10). Although there has been some work on the roles of NF-κB and MAPKs in the regulation of MMP-9 production by LPS, little is known of the involvement of reactive oxygen species (ROS) in the pathways leading to MMP-9 expression in monocytes/macrophages.

A growing body of evidence points to a role of oxygen-derived radicals such as superoxide anion (\(O_2^-\)), hydrogen peroxide (\(H_2O_2\)), and NO as intracellular signaling molecules (11, 12). For example, ROS can affect the expression of a number of genes in monocytes. \(H_2O_2\) is reported to activate NF-κB, which regulates the expression of proinflammatory cytokines and immune mediators (13). In addition, C6-ceramide induces ROS production, leading to apoptosis via the transcription factor AP-1 (14). NADPH oxidase activation during phagocytosis is an important source of ROS in phagocytes, including macrophages. NADPH oxidase is inhibited by diphenyleneiodonium chloride (DPI) (15), and a recent report suggests that DPI is also a potent inhibitor of mitochondrial ROS.
ROS production in monocytes/macrophages (16). Moreover, it has been established that mitochondrial ROS induce NF-κB, AP-1, and HIF-1 DNA binding activity in monocytes, lymphocytes, and hepatocytes, respectively (17–19). Consistent with these findings, mitochondrial ROS was suggested to mediate gene (e.g., angiotensinogen gene) expression in response to high glucose in kidney tubular cells (20). Because mitochondria generate ROS at the ubisemiquinone site, a change in mitochondrial redox could affect ROS production. We report in this paper that the induction of MMP-9 expression by LPS in Raw 264.7 cells is mediated by a mitochondria-derived ROS-p38 kinase cascade that leads to stimulation of AP-1.

Materials and Methods

Reagents

BSA, DMSO, gelatin, LPS, and PMA were obtained from Sigma-Aldrich (St. Louis, MO). The following were obtained from Calbiochem (La Jolla, CA): GF109203X, a general PKC inhibitor ($IC_{50}$ = 0.2 μM) (21); Go6976, a potent inhibitor of Ca$^{2+}$-dependent PKC isozymes ($IC_{50}$ = 6.2 nM) (21); rottlerin, a PKCδ inhibitor ($IC_{50}$ = 3–6 μM) (22); calphostin C, a diacylglycerol-dependent PKC inhibitor ($IC_{50}$ = 50 nM) (23); PD98059, a MAPK kinase inhibitor ($IC_{50}$ = 2 μM) (24); SB203580, a p38 inhibitor ($IC_{50}$ = 0.6 μM) (25); SP600125, a JNK inhibitor ($IC_{50}$ = 0.19 μM) (26); pyrrolidine dithiocarbamate (PDTC), a NF-κB inhibitor (27); and rotenone, a mitochondrial complex I inhibitor ($IC_{50}$ = 8–20 nM) (28). BSA, DDEM, and nonessential amino acids were obtained from Invitrogen Life Technologies (Gaithersburg, MD). Abs to p38 kinase were supplied by New England Biolabs (Beverley, CA), and Abs to MMP-9, PKCδ, and phospho-PKCδ were obtained from Cell Signaling Technology (Beverly, MA). All other chemicals were obtained from standard sources and were molecular biology grade or higher.

Cell culture

Murine Raw 264.7 macrophage cells (American Type Culture Collection, Manassas, VA; CRL 2278) were grown in DMEM supplemented with 10% heat-inactivated FBS, 0.1 mM nonessential amino acids, penicillin (50 U/ml), and streptomycin (50 μg/ml) at 37°C under a humidified 95/5% mixture of air and CO2.

Assay of MMP-9 activity by gelatin zymography

MMP-9 activity was assessed by gelatin zymography. Briefly, we cultured cells (2 x 105 cells/12 well) for 12 h, collected culture supernatants, added Laemmli buffer without reducing agent to the supernatants, and subjected absence of EDTA and stained with Coomassie Brilliant Blue solution.

Measurement of MMP-9 by Western blot analysis

Levels of MMP-9 protein in culture supernatants and cell lysates were measured by Western blot analysis. Briefly, culture supernatants and cell lysates were heated at 95°C for 5 min, subjected to 8% SDS-PAGE, and transferred to polyvinylidene difluoride membranes with incubation overnight at 4°C in Tris buffer containing nonfat dried milk (5%, w/v) and Tween 20 (0.01%, v/v). Subsequently, the membranes were incubated with the appropriate Abs, followed by second Ab (HRP-conjugated Ab), and developed by ECL, as instructed by the manufacturer (Amersham Biosciences, Arlington Heights, IL).

Infection of Raw 264.7 cells with retroviral dominant negative PKCδ (DN-PKCδ)

Retroviral expression vectors for vector and a kinase-deficient form of PKCδ (PKCδ-K376R) that acts a DN mutant were provided by Dr. S. J. Lee (Korea Institute of Radiological and Medical Sciences, Seoul, Korea). Raw 264.7 cells were infected with these vectors and seeded in 35-mm culture plates in DMEM supplemented with 10% FBS. pMFG and pMFG-DN-PKCδ were incubated with the retrovirus for 3 h at 37°C in serum-free DMEM. An equal volume of DMEM with 20% FBS was then added to increase the serum concentration to 10%, and incubation was continued for a total of 36 h. Thereafter, the cells were exposed to LPS and assayed for MMP-9, and levels of PKCδ were determined by Western blotting.

ROS measurement

Intracellular H2O2 was measured by 2′,7′-dichlorofluorescin fluorescence (29, 30). Briefly, Raw 264.7 cells were grown for 24 h and then serum-starved for 6 h in phenol red-free DMEM supplemented with 0.5% FBS. To measure intracellular H2O2, the cells were then incubated for 30 min with the H2O2-sensitive fluorophore 2′,7′-dichlorofluorescin diacetate (DCFH-DA; 10 μg/ml), which is taken up and oxidized to the fluorescent DCF by intracellular H2O2. After incubation with DCFH-DA, the cells were exposed to LPS for 30 min and immediately observed by FACS Calibur (BD Biosciences, Franklin Lakes, NJ). To measure mitochondria-generated ROS, fluorescence was excited at 488 nm, and the evoked emission was filtered with a 515-nm longpass filter.

Subcellular fractionation for mitochondrial O2 measurement

Cells were stimulated with 100 ng/ml LPS for 20 min, washed three times with ice-cold PBS, and scraped into ice-cold HES buffer (255 mM sucrose, 20 mM HEPES (pH 7.4), and 1 mM EDTA) supplemented with 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, and protease inhibitors (1 μg/ml leupeptin, 1 μg/ml peptatin, 1 mg/ml benzamidine, and 0.5 mM PMSF). Cell lysates were fractionated into plasma membrane and mitochondrial/nuclear fractions by differential centrifugation. Mitochondria-enriched intracellular membranes were released from washed mitotic cells by mechanical homogenization or freeze-thaw permeabilization and collected in the supernatant after centrifugation at 15,000 x g for 5 min. For mechanical homogenization, cells were passed through a 21-gauge needle. Superoxide production was detected by luminometer as lucigenin-derived chemiluminescence. It was measured in the presence of NADPH for 15 min and expressed as mean arbitrary light units per minute (16).

Transient transfection and reporter gene assays

Cells (2 x 105/35-mm plate) were cultured for 24 h and incubated with Lipofectamine and DNA complex (1.8 μg of DNA/plate) for 4 h. Sonicated calf thymus DNA was used to keep the concentration of DNA in each transfection mixture constant. To monitor variations in cell numbers and transfection efficiency, all clones were cotransfected with 0.4 μg of pSV40-β-galactosidase, a eukaryotic expression vector containing the Escherichia coli β-galactosidase (lacZ) structural gene under control of the SV40 promoter. After incubation with the Lipofectamine/DNA complex for 4 h, the cells were rinsed with PBS, incubated for 24 h in DMEM with 10% FBS, rinsed twice more with PBS, and lysed with 0.1 ml of lysing solution (0.2 M Tris-Cl with 0.1% Triton X-100, pH 7.6). The lysed cells were scraped off and spun for 1 min, their supernatants were collected, and protein and β-galactosidase and luciferase activities were measured in the supernatants. Luciferase luminescence was measured with a luminometer (Turner, Palo Alto, CA; TD-20/20) and normalized to the cotransfected β-galactosidase activity as previously described (30). Data are the averages of triplicate independent determinations.

Statistical analysis

Data are expressed as the mean percentage ± SD. Statistical comparisons between groups were made with Student’s t test. Values of p < 0.05 were considered significant.

Results

LPS induces expression of biologically active MMP-9 in Raw 264.7 cells

To determine whether LPS induces expression of MMP-9 in Raw 264.7 murine macrophage, cells were cultured in the presence of LPS, and the level of MMP-9 protein in the culture medium was determined. MMP-9 protein was not detected in supernatants of control cells cultured without LPS, whereas supernatants of LPS (12 h)-treated cells contained a substantial amount of MMP-9 protein as defined by Western blot analysis (Fig. 1, upper panel). To determine whether the MMP-9 protein released in response to LPS was biologically active, we assessed gelatinolytic activity in the culture supernatants. As shown in Fig. 1, gelatinolytic activity was not detected in the control supernatant, whereas the supernatant of LPS-treated cells had strong gelatinolytic activity 12 h post-LPS treatment (Fig. 1, lower panel). In contrast, the activity of MMP-2, which is constitutively expressed, was unchanged by LPS treatment.
LPS induces MMP-9 expression via a p38 kinase pathway

Because MAPKs have been implicated in the induction of MMPs (31, 32), we examined the effect of LPS on MAPK activation. Cells were treated with LPS in the presence of various MAPKs inhibitors, and MMP-9 activity was assessed. As shown in Fig. 2, inhibition of p38 kinase with SB203580 (a p38 kinase inhibitor) reduced LPS-induced MMP-9 expression, and in agreement with these results, we found that LPS stimulated p38 kinase activity as detected by phosphorylation of p38 kinase. In contrast, LPS-induced activation of p38 kinase and MMP-9 activity was not affected by PD98059 (an ERK kinase inhibitor), or SP600125 (a JNK kinase inhibitor), suggesting that p38 kinase has a specific role in signaling MMP-9 expression.

Rottlerin inhibits LPS-induced MMP-9 expression

Next we examined the pathway upstream of p38 kinase activation. Because in certain cases induction of MMP-9 has been reported to be impaired by inhibition of PKCs (31, 32), we examined the involvement of PKCs in our system. Cells were treated with LPS alone or together with various PKC inhibitors, and MMP-9 expression was measured. Various inhibitors, such as rottlerin, were used. Rottlerin, a putative inhibitor of PKCδ, significantly attenuated LPS-induced MMP-9 expression as well as p38 kinase phosphorylation, whereas other PKC inhibitors, Ro-32-0432, Go-6976, GF-109203X, and calphostin C, had no effect (Fig. 3A). As shown in Fig. 3B, LPS-induced MMP-9 expression and p38 kinase phosphorylation were reduced in a dose-dependent manner by rottlerin. These observations led us to hypothesize that PKCδ acts upstream of p38 kinase in the pathway from LPS to MMP-9 expression in Raw 264.7 cells.

LPS induces MMP-9 expression is independent of PKCδ

To further examine the role of PKCδ in LPS signaling to MMP-9 expression, Raw 264.7 cells were exposed to PMA for 12 h to deplete PKCδ and other members of the novel PKC subfamily as well as of the conventional subfamily. Immunoblot analysis revealed that PKCα, PKCδ, and PKCζ were strongly down-regulated by the prolonged exposure to PMA, whereas PKCβi was unaffected (Fig. 4A). To our surprise, MMP-9 expression was not affected by this prolonged PMA treatment (Fig. 4B), suggesting that the induction of MMP-9 by LPS is independent of both conventional and novel isotypes of PKC, including PKCδ. To confirm that LPS induces MMP-9 expression independently of PKCδ, cells were infected with a retrovirus encoding a kinase-deficient form of PKCδ (Fig. 5A).

FIGURE 1. LPS induces MMP-9 expression in Raw 264.7 cells. Raw 264.7 cells were incubated for the indicated periods with increasing concentrations of LPS in DMEM containing 0.5% FBS. At the times indicated, cell lysates were fractionated on 7.5% SDS-PAGE to determine the level of MMP-9 protein expression (upper panel), and the gelatinolytic activity of MMPs was determined by zymography as described in Materials and Methods (lower panel). MMP-2 is shown as a control. The data shown are representative of three independent experiments.

FIGURE 2. LPS induces MMP-9 expression via a p38 kinase-dependent pathway. Upper panel. Cells were pretreated with 10 μM PD98059, 10 μM SB203580, or 10 μM SP600125 for 30 min before application of LPS for 12 h. Twenty microliters of the conditioned medium was subjected to 7.5% SDS-PAGE containing 0.2% gelatin, and the gelatinolytic activity of the MMP was determined by zymography as described in Materials and Methods. Lower panel, p38 kinase phosphorylation triggered by 100 ng/ml LPS for 20 min was assayed after exposing the cells to PD98059, SB203580, or SP600125 for 30 min. Immunoblots of whole cell extracts were probed with anti-phospho-p38 Ab, after which they were stripped and reprobed with anti-p38 Ab. The results are representative of three separate experiments.

FIGURE 3. LPS is unable to induce MMP-9 expression in the presence of rottlerin. A. Cells were pretreated with rottlerin (2 μM), Ro-32-0432 (0.2 μM), Go-6976 (0.1 μM), GF-109203X (0.1 μM), and calphostin C (0.1 μM) for 30 min before challenge with LPS for 12 h (zymography) or 20 min (immunoblots). Conditioned media and whole-cell lysates were assayed for gelatinolytic activity by zymography and for p38 phosphorylation by Western blotting. B. Cells were incubated in DMEM containing 0.5% FBS with LPS (100 ng/ml) in the presence or the absence of the indicated concentration of rottlerin (0, 2, 5, and 10 μM). The results shown are representative of three independent experiments.
PKC\textsubscript{H9254}, PKC\textsubscript{H9254}-K376R, that acts as a DN mutant (33). As shown in Fig. 4B, cells infected with this virus accumulated MMP-9 as efficiently as the control cells (Fig. 4B), again ruling out the involvement of PKC\textsubscript{H9254}. Clearly, the effect of rottlerin on MMP-9 expression is not due to inhibition of PKC\textsubscript{H9254}.

**LPS induces p38 kinase and MMP-9 via a ROS pathway**

There are reports that rottlerin is a putative mitochondrial uncoupler as well as a PKC\textsubscript{H9254} inhibitor (34–36) and thus potentially affects mitochondrial O\textsubscript{2}/H\textsubscript{6107} production. We next tested any role of ROS for the expression of MMP-9 and the phosphorylation of p38 kinase in response to LPS. The NADPH oxidase-like flavoenzyme inhibitor DPI was found to block LPS-induced MMP-9 expression and p38 kinase phosphorylation (Fig. 5A). Similarly, the ROS scavenger N-acetylcysteine (NAC) reduced both MMP-9 expression and p38 kinase phosphorylation, suggesting that ROS act upstream of p38 kinase in LPS signaling to MMP-9 expression. However, PDTC, which primarily inhibits NF-\kappaB, had no inhibitory effect (Fig. 5A). Next, we examined the effect of LPS on levels of ROS using the DCF-DA assay. This assay measures H\textsubscript{2}O\textsubscript{2} indirectly by measuring the DCF generated by H\textsubscript{2}O\textsubscript{2} from DCF-DH. LPS indeed enhanced DCF fluorescence, and this effect was dramatically inhibited by rottlerin (Fig. 5B). It thus appears that LPS stimulates the generation of ROS, and this leads to p38 kinase activation and subsequently to MMP-9 expression.

**LPS induces MMP-9 through a mitochondrial ROS-dependent pathway**

Besides acting as an NADPH oxidase-like flavoenzyme inhibitor, DPI has also been reported to inhibit mitochondrial complex I (NADH-ubiquinone oxidoreductase), so decreasing oxidative phosphorylation (37). In addition, rotenone and DPI strongly inhibit mitochondrial ROS production in monocytes/macrophages (16, 38). We therefore examined the possible role of mitochondrial ROS in LPS-induced MMP-9 expression. When cells were treated with LPS together with the mitochondrial complex I inhibitor rotenone, little phospho-p38 kinase or MMP-9 was detected. In contrast, the mitochondrial complex II inhibitor thenoyltrifluoroacetone (TTFA) had no effect, and the mitochondrial complex III inhibitor antimycin A had only a slight effect (Fig. 6A). These results suggest that LPS somehow acts primarily through the mitochondrial complex I ROS.

To determine whether LPS activates mitochondrial ROS production, we used the DHR assay that is more selective for mitochondrial-derived ROS (39). As shown in Fig. 6B, LPS increased DHR fluorescence in the control cells, whereas there was no increase in the presence of the mitochondrial complex I inhibitor rotenone. To our surprise, rottlerin also attenuated DHR fluorescence, suggesting its potential activity as an antioxidant. Similar to...
these results, in lucigenin-derived chemiluminescence assays that detect only intramitochondrial $O_2^-$ production (16), pretreatment with rotenone and rottlerin completely abolished LPS-induced $O_2^-$ production in a mitochondria-enriched membrane fraction (Fig. 6C), further substantiating that LPS activates mitochondrial ROS production. Consistent with the idea that p38 kinase acts downstream of ROS in LPS signaling to MMP-9 expression, the p38 kinase inhibitor SB203580 had no effect on ROS levels (Fig. 6B).

**LPS activates AP-1 activity in a ROS/p38 kinase-dependent manner**

We next examined the signaling events after p38 kinase activation. It has been reported that MAPKs activate NF-κB and AP-1 and that the MMP-9 promoter contains NF-κB- and AP-1-responsive elements (9, 10). To determine whether LPS activates AP-1 promoter activity, Raw 264.7 cells were transfected with an AP-1-dependent luciferase reporter gene, and their response to LPS was assessed. LPS significantly increased AP-1-dependent luciferase activity, and this effect was severely reduced by pretreatment with rottlerin or SB203580 (Fig. 7). In contrast, PDTC failed to reduce LPS-dependent luciferase activity. Clearly, LPS-dependent activation of AP-1 is correlated with LPS-dependent MMP-9 expression.

**LPS induction of MMP-9 expression is independent of NF-κB**

Next, we examined the effect of LPS on the activation of NF-κB. LPS clearly induced IkBα degradation and consequent NF-κB stimulation, but pretreatment with rotenone, TTFA, or antimycin A did not inhibit these processes (Fig. 8A), nor did rottlerin or SB203580 (data not shown). Therefore, LPS-induced mitochondrial ROS generation and p38 kinase activation are independent of NF-κB. We also transfected cells with an NF-κB-dependent luciferase reporter gene and found that LPS induced NF-κB luciferase activity essentially as efficiently in the presence of rottlerin or SB203580 as in controls (Fig. 8B). In contrast, it was unable to stimulate NF-κB luciferase activity in the presence of PDTC, an NF-κB inhibitor (Fig. 8B). These results together with the earlier data (Fig. 5A) indicate that NF-κB is not involved in the LPS-induced ROS/p38 kinase cascade leading to MMP-9 expression.
Inhibition of tyrosine phosphorylation blocks the secretion of proinflammatory cytokines and MMP-9 (47). Previous reports have shown that LPS increases MMP-9 production by activating the ERK subgroup of MAPKs as well as p38 kinase in monocytes, astrocytes, and neutrophils (48–50).

Initially, we thought, based on the results obtained with rottlerin, a putative PKC inhibitor, that PKC participated in LPS-induced MMP-9 expression. However, because long term PMA treatment and DN-PKC8 overexpression did not affect LPS-induced MMP-9 expression (Fig. 4), we concluded that the inhibitory effect of rottlerin on MMP-9 expression was independent of PKC8. Importantly, we were able to show that pretreatment with DPI, NAC, or rotenone, a specific inhibitor of mitochondrial complex I, dramatically reduced LPS-induced p38 kinase phosphorylation and MMP-9 expression (Figs. 5A and 9). Because DPI and rotenone have also been shown to inhibit mitochondrial complex I activity and reduce mitochondrial ROS production (16, 37, 38), it is conceivable that mitochondrial ROS are downstream components of LPS signaling. In agreement with this idea, we showed that LPS increased ROS levels at that pretreatment with rotenone completely blocked that effect (Fig. 5B and Fig. 6B and C). It is puzzling that rottlerin reduces mitochondrial ROS levels, because uncouplers would be expected to increase O2 consumption and the flux of electrons through the electron transport system, thereby increasing the production of ROS at the level of reduction of molecular oxygen. However, the precise action of mitochondrial uncouplers on ROS production remains to be clarified. Interestingly, Parthasarathi et al. (38) reported that carbonyl cyanide p-trifluoromethoxyphenylhydrazone, another type of mitochondrial uncoupler, strongly inhibits mitochondrial Ca2+ and ROS generation in response to TNF-α; this suggests that the effect of an uncoupler on levels of mitochondrial ROS could vary with the cell type or agonist used. In addition, we cannot rule out the possibility that the inhibitory effect of rottlerin on mitochondrial ROS is due to an antioxidant action, independent of any activity as an uncoupler. In any event, to determine whether other reactive species, e.g., NO or NADPH oxidase-derived ROS, are also involved in LPS signaling to MMP-9, we tested the effects of N’-monomethyl-L-arginine, a NO synthase and NADPH oxidase inhibitor, on this pathway. We found that it had no effect, suggesting that NO is not involved (data...
Mitochondrial ROS have been implicated in signaling in response to a variety of treatments that induce gene transcription (20, 38), and other workers have also shown that p38 kinase is activated by intracellular ROS as well as by exogenously administered H$_2$O$_2$ (51–54). Hypoxia can also lead to p38 kinase activation (55), although neither the mechanism underlying this activation nor its relationship to ROS has been clarified. In the case of hypoxia, mitochondrial ROS are thought to be responsible for triggering p38 kinase phosphorylation and gene expression (55). Mitochondrial ROS production can be harmful, because it abolishes the mitochondrial potential and leads to the release of cytochrome c and apoptosis (14, 56). However, as far as we could determine, the levels of ROS attained in our experiments were not cytotoxic (data not shown), suggesting that the LPS-induced mitochondrial ROS production observed is an intrinsic, not a pathological, feature of cell signaling. The mechanism by which ROS induces p38 kinase activation is not clear, but a caspase linked to mitochondrial ROS and p38 kinase is also believed to mediate angiotensinogen expression in response to high glucose in kidney tubule cells (20).

We also examined the nuclear targets of activated p38 kinase. Transcription of MMP-9 is usually regulated by inflammatory cytokines such as TNF-α and IL-1, whereas MMP-2 is constitutive in many cell types (57, 58). The MMP-9 promoter has both NF-κB and AP-1 binding sites, whereas the MMP-2 promoter does not contain any AP-1 binding sites, and this difference may be related to their differential regulation (59). Usually, ROS production in response to external stimuli is associated with activation of AP-1 and NF-κB. To assess how ROS generated in mitochondria contribute to the LPS signaling, we measured NF-κB and AP-1 activity in response to LPS. Because NF-κB has been suggested to be a key transcription factor regulating MMP-9 expression (59–63), we asked whether LPS induces MMP-9 via the NF-κB pathway in addition to activating p38 kinase. Our data show that LPS-dependent activation of NF-κB is unrelated to the activation of ROS and p38 kinase. We also demonstrated that rotterlin, rotenone, and SB203580 had no inhibitory effect on LPS-induced iNOS degradation and NF-κB reporter gene activity, whereas PDTC inhibited it strongly, indicating that the ROS-p38 kinase-linked cascade represents an alternative signaling pathway leading to MMP-9 expression that does not involve NF-κB (Fig. 8). Likewise, application of rotterlin and SB203580 reduced the activation of AP-1 by LPS (Fig. 7) and also strongly inhibited LPS-induced MMP-9 promoter activity in the Raw 264.7 cells, indicating that the mitochondrial ROS-p38 kinase pathway regulates LPS-induced MMP-9 expression via AP-1 (Fig. 7).

Similar to our results, the p38 kinase signal transduction pathway has been shown to stimulate MMP-9 expression in an AP-1-dependent fashion in human vascular smooth muscle cells (64); more importantly, Sano et al. (51) have reported that IL-6 is induced by angiostatin II in cardiac fibroblasts via a ROS-p38 kinase-dependent, not an NF-κB-dependent, pathway. Hence a mitochondrial ROS-p38 kinase-AP-1 cascade may exist in certain cell types in addition to Raw 264.7 cells, mediating specific signaling reactions in response to a limited number of agonists. The reason why this cascade is confined to certain cases is not clear, but may be related to the unique reaction of certain cell types, including monococytes, to mitochondrial electron transport inhibitors such as rotenone and antimycin A.

Recently, Yoo et al. (60) reported that IL-1β up-regulates MMP-9 expression in RAW 264.7 cells via the production of ROS and subsequent activation of NF-κB. However, we could not detect any MMP-9 up-regulation in response to IL-1β in our experimental conditions (data not shown). Those authors did not show by zymography or Western blotting that NF-κB inhibitors such as PDTC actually blocked IL-1β-induced MMP-9 up-regulation (60). They did observe that NAC inhibited MMP-9 promoter activity, but it may also have inhibited activation of AP-1, as suggested by our results. As in our case, Yoo et al. (60) observed that DCF-sensitive ROS production played a critical role in the signaling pathway to MMP-9 expression, although the source of the ROS was not defined. In view of the undoubted role of the mitochondrial ROS-p38-AP-1 pathway in MMP-9 up-regulation in response to LPS in the present study, we speculate that the ROS detected by Yoo et al. (60) was also mitochondrial.

In conclusion, we showed that redox-regulated p38 phosphorylation and subsequent AP-1 activation appear to be critical for LPS-induced MMP-9 expression, at least in Raw 264.7 cells. Future studies elucidating the linkage between mitochondrial ROS-p38 kinase and AP-1 will probably be pivotal to our complete understanding of LPS-evoked intracellular signaling to MMP-9 expression and inflammatory response.

References


