Participation of Reactive Oxygen Species in the Lysophosphatidic Acid-stimulated Mitogen-activated Protein Kinase Kinase Activation Pathway*

(Received for publication, August 31, 1995, and in revised form, October 5, 1995)

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Recent evidence suggests that reactive oxygen species (ROS) may function as second messengers in intracellular signal transduction pathways. We explored the possibility that ROS were involved in lysophosphatidic acid (LPA)-induced mitogen-activated protein (MAP) kinase signaling pathway in HeLa cells. Antioxidant N-acetylcysteine inhibited the LPA-stimulated MAP kinase activation pathway. Direct exposure of HeLa cells to hydrogen peroxide resulted in a concentration- and time-dependent activation of MAP kinase kinase. Inhibition of catalase with aminotriazole enhanced the effect of LPA on induction of MAP kinase kinase. Further, LPA stimulated ROS production in HeLa cells. These findings suggest that ROS participate in the LPA-elicited MAP kinase signaling pathway.

Reactive oxygen species (ROS),¹ such as superoxide (O₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radical (OH·), are potent microbicidal agents, but excess ROS can also cause oxidative damage to macromolecules of host cell (1). Previous studies have shown that elevated levels of ROS could trigger intracellular signaling transduction pathways that may mediate cellular protective responses (2–4). In addition to their roles in inflammatory and pathological processes, increasing evidence suggests that ROS may function as second messengers in cytokine (interleukin-1 and tumor necrosis factor α) and some growth factor signal transduction pathways that regulate transcription factors such as NF-κB and AP-1 (5, 6).

Lysophosphatidic acid (LPA) is released by activated platelets and is thought to be responsible for much of the activity in serum that promotes cell growth and adhesion (7, 8). LPA elicits its biological responses through a putative receptor that is coupled to heterotrimeric G-proteins (9). Several proximal signaling events are known to be evoked by LPA, including phosphoinositide hydrolysis and Ca²⁺ mobilization, release of arachidonic acid, inhibition of adenylate cyclase, and induction of protein tyrosine phosphorylation (10, 11). It is likely that some of these signaling events cross-interact to induce synergistic responses.

LPA rapidly activates the mitogen-activated protein (MAP) kinase pathway (11–14). MAP kinases are serine/threonine-protein kinases regulated by dual tyrosine and threonine phosphorylation. Three subfamilies of MAP kinases, MAPK, JNK, and HOG, have been cloned (15–17). The mitogen-activated protein kinase kinase 1 and 2 (MKK1/2) was determined by the following molecular oncology program and the cell biology program, H. Lee Moffitt Cancer Center and Research Institute, MDC Box 44, 12902 Magnolia Dr., Tampa, FL 33612. Tel.: 813-979-6713; Fax: 813-979-3893.

1 The abbreviations used are: ROS, reactive oxygen species; MAP kinase, mitogen-activated protein kinase; MKK1/2, MAP kinase kinase 1 and 2; BSA, bovine serum albumin; LPA, lysophosphatidic acid; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter; EGF, epidermal growth factor.

2 Q. Chen and J. Wu, unpublished data.

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Vol. 270, No. 48, Issue of December 1, pp. 28499–28502, 1995
Printed in U.S.A.
Fig. 1. Inhibition by N-acetylcysteine on MKK1/2 activation.

Serum-starved HeLa cells were treated with or without N-acetylcysteine (NAC, 30 mM) for 90 min, and left unstimulated (−) or stimulated with lysophosphatidic acid (20 μM) or EGF (25 ng/ml) for 5 min. MKK1/2 activity was determined using a kinase-defective p42^mapk (K52R) as substrate. The kinase reaction was carried out at 30 °C for 15 min. Arrow, K52R band.

Fig. 2. Activation of MKK1/2 by H_2O_2. Serum-deprived HeLa cells were treated without (−ATZ) or with 50 mM aminotriazole (+ATZ) for 60 min followed by stimulation for 5 min with different concentrations of H_2O_2 as indicated (A) or with 1 mM H_2O_2 in the absence of aminotriazole for the indicated time (B). Total activity of MKK1/2 was determined. The phosphorylation of K52R was quantitated with a PhosphorImager (Molecular Dynamics) after SDS-gel electrophoresis. The basal activity (21 pmol/min/mg) of MKK1/2 in unstimulated cells was arbitrarily set as 1 unit.

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RESULTS AND DISCUSSION

In all cell types examined, p42^mapk is specifically phosphorylated and activated by dual specificity MKK1 and MKK2 (MKK1/2). Total activity of MKK1/2 in the cells represents a valid measurement for the activation state of the MAP kinase pathway in the rapid activation phase (14, 23). As illustrated in Fig. 1, LPA and EGF markedly stimulated the MKK1/2 activity in HeLa cells. To test for the possible involvement of ROS in MKK1/2 activation induced by LPA, we examined the effect of antioxidant N-acetylcysteine on MKK1/2 activation. N-Acetylcysteine directly scavenges ROS and also increases the intracellular levels of reduced glutathione (GSH). GSH is a hydroxyl radical scavenger and a substrate of glutathione peroxidase which degrades H_2O_2. N-Acetylcysteine has been used extensively to study the role of ROS in signaling pathways (6, 25–27). An inhibition by N-acetylcysteine can be taken as an indication of the involvement of ROS. The LPA-stimulated MKK1/2 activity was inhibited by 82 ± 4% (average of two experiments ± range) in cells pretreated with N-acetylcysteine (30 mM), suggesting that ROS are involved in the LPA-induced MKK1/2 activation (Fig. 1). A similar result was obtained in Rat-1 cells. A lesser, but statistically significant, attenuation (38 ± 8% in two experiments) by N-acetylcysteine of the EGF-stimulated MKK1/2 activity was also observed (Fig. 1), but was not investigated further in the current study.

To verify that the inhibitory effect of N-acetylcysteine is attributable to its ability to scavenge ROS, we examined the effects of two other ROS scavengers, dimethyl sulfoxide and ascorbic acid, on the LPA-stimulated MKK1/2 activity. Dimethyl sulfoxide is an effective hydroxyl radical scavenger (28). Ascorbic acid blocks free radical chain reaction, but may also directly remove hydroxyl radical (29). HeLa cells were pretreated with ascorbic acid (100 μM, 60 min) or dimethyl sulfoxide (4%, 20 min) and stimulated with LPA (10 μM, 5 min). The LPA-stimulated MKK1/2 activity was inhibited by 88 ± 7% by dimethyl sulfoxide and 38 ± 1% by ascorbic acid.

If ROS are the signaling molecules that mediate the LPA-induced MKK1/2 activation, then an increase in intracellular concentrations of ROS would be expected to mimic the effect of LPA on MKK1/2 activation. H_2O_2 is the product of superoxide dismutases and several oxidases in the cells. Thus, cells that produce superoxide would also generate H_2O_2. In contrast to superoxide, H_2O_2 can diffuse across the membrane and give rise to the highly reactive hydroxyl radical. H_2O_2 has been widely used to assess the role of ROS in cells. To test whether H_2O_2 directly added to the cells can activate MKK1/2, HeLa cells were treated with 0.1–4 mM H_2O_2 for 5 min or with 1 mM H_2O_2 for 2.5–30 min, and the MKK1/2 activity was determined. Fig. 2 shows that H_2O_2 caused a concentration- and time-dependent activation of MKK1/2 in HeLa cells. Thus, H_2O_2 alone is sufficient to induce MKK1/2 activation. The maximal activity of MKK1/2 induced by H_2O_2 in HeLa cells was detected approximately 5 min after treatment. Thus, the kinetics of H_2O_2 induction is similar to that of MKK1/2 activation induced by phospholipids and growth factors (13, 30). However, 2 mM H_2O_2, a concentration of H_2O_2 that cannot be achieved in HeLa cells by LPA (data not shown), is required to induce MKK1/2 activation to a similar magnitude (approximately 20-fold) as 20 μM LPA.

Several possibilities exist that may account for the requirement of high concentrations of H_2O_2. First, the LPA-induced ROS may be generated at a site that is more proximal to the...
target, whereas the external added H$_2$O$_2$ diffuses indiscriminately. Second, HeLa cells may contain relatively high catalase activity, and, thus, high concentrations of H$_2$O$_2$ are required to offset the catalase activity. In fact, inhibition of catalase by preincubation of HeLa cells with catalase inhibitor aminotriazole (26) prior to the addition of H$_2$O$_2$ resulted in a marked shift of the H$_2$O$_2$ dose-response curve to the left (Fig. 2A). However, even in the presence of aminotriazole, greater than 0.5 mM H$_2$O$_2$ was still required to activate MKK1/2 to a similar extent as that induced by 20 mM H$_2$O$_2$. Finally, it is likely that one or more signaling events besides production of ROS are critical for the induction of MKK1/2 activation by LPA, and ROS may function as only one of the parallel signaling intermediates. Thus, although H$_2$O$_2$ alone at low concentrations (<0.5 mM) has a marginal effect on MKK1/2 activation, H$_2$O$_2$ and the derived radicals may have a greater effect in the presence of other LPA-induced signaling intermediates because of synergism.

To further confirm the involvement of ROS, we treated HeLa cells with or without the catalase inhibitor aminotriazole prior to LPA stimulation. If ROS participate in the LPA-stimulated MKK1/2 activation pathway, inhibition of catalase would potentially augment the response to LPA. A 4.5-fold increase in H$_2$O$_2$-induced MKK1/2 activity was observed when HeLa cells were pretreated with aminotriazole (50 mM, 60 min), demonstrating the effectiveness of the catalase inhibitor (Fig. 3, see also Fig. 2A). In cells pretreated with aminotriazole, the LPA-stimulated MKK1/2 activity was 1.9-fold that of cells without aminotriazole pretreatment (36.3- and 18.8-fold above basal, respectively) (Fig. 3). Thus, decreasing the catalase activity effectively enhances the cellular response to LPA, indicating the involvement of ROS.

For ROS to fulfill the role of signaling intermediates for LPA, LPA must be able to induce the production of ROS. As described above, LPA rapidly liberates arachidonic acid in HeLa cells. Arachidonic acid is known to generate ROS. Tumor necrosis factor $\alpha$ and interleukin-1, both of which are known to utilize ROS as signaling intermediates, also stimulate the release of arachidonic acid (31). However, other routes of ROS generation are not excluded. We measured the relative concentrations of H$_2$O$_2$ in HeLa cells using dihydorhodamine 123 and fluorescence-activated cell sorting (FACS) (6, 24). Dihydorhodamine 123 is oxidized to membrane-impermeable, fluorescent rhodamine 123 in the presence of H$_2$O$_2$ and possibly ROS derived from it (24). To minimize the loss of H$_2$O$_2$, aminotriazole was also added to the media. Incubation of HeLa cells with aminotriazole resulted in a time-dependent increase in fluorescence intensity (Fig. 4 and data not shown). A small increase of the fluorescence intensity induced by LPA was detectable at the earliest time (5 min) examined, but more consistent data were obtained if cells were stimulated for 10 min. In two duplicated experiments, cells treated for 10 min with aminotriazole (50 mM) plus BSA had an average 25% increase in mean fluorescence intensity of rhodamine 123 compared with BSA-treated cells (Fig. 4). An additional 22% increase in mean fluorescence intensity was detected in cells treated for 10 min with LPA (30 $\mu$M) plus aminotriazole (50 mM) (Fig. 4). Thus, LPA is capable of generating ROS in HeLa cells.

In summary, data presented in this study show that ROS are involved in the LPA-induced MAP kinase kinase activation and LPA can stimulate the production of ROS in HeLa cells. Additional experiments using a p42$\text{mapk}$ immune complex kinase assay (14) showed that N-acetylcysteine partially inhibited the LPA-stimulated p42$\text{mapk}$ activation, H$_2$O$_2$ stimulated p42$\text{mapk}$ activity in HeLa and NIH 3T3 cells, and aminotriazole enhanced the effect of LPA on p42$\text{mapk}$ activation (data not shown). Previous studies have shown that ROS are involved in the tumor necrosis factor $\alpha$-stimulated NF-$\kappa$B activity (5) and the basic fibroblast growth factor-induced c-fos expression (6).

Other data that we have obtained showed that N-acetylcysteine also inhibited the LPA-stimulated NF-$\kappa$B and AP-1 DNA binding activities in HeLa cells. Thus, ROS appear to function as signaling intermediates of LPA and mediate a branch of the LPA signaling pathways. Our findings lend support to the emerging concept that ROS can function as physiological signaling intermediates. It will be interesting to examine whether ROS also participate in the signaling pathways of other phospholipids, such as platelet-activating factor (32) and sphingosine 1-phosphate (33). Clearly, further investigation of the mechanisms by which LPA increases the intracellular levels of ROS and ROS relay the cellular regulatory signals is warranted.

Acknowledgments—We thank Christine O’Connell for the FACS analysis and Drs. Warren J. Pledger and W. Douglas Cress for critical reading of the manuscript.

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