Selective Activation of p38 MAPK Cascade and Mitotic Arrest Caused by Low Level Oxidative Stress*

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Apoptosis induced by high level oxidative stress accompanies diverse cellular biochemical events including activation of the stress signal cascades of JNK and NF-κB. We report here selective activation of p38 MAPK cascade and mitotic arrest under a low level oxidative stress that lacks apoptosis induction. U937 human lymphoid cells treated with low dose (0.02 mM) H2O2 rapidly caused p38 MAPK cascade activation detectable by phosphorylation of MKK3/6, p38 MAPK, activating transcription factor-2, and cAMP-responsive element-binding protein, leaving the JNK and NF-κB cascades unaffected. The p38 kinase activation was sustained for 24 h under the low level stress conditions and led to formation of polyploid nuclei. N-Acetyl-l-cysteine, a precursor of anti-oxidant glutathione, canceled both p38 MAPK activation and abnormal cell cycle progression, whereas blockage of the kinase by specific inhibitor SB203580 allowed the appearance of apoptotic cells. Thus, mimicking the effects of nocodazole, the low level oxidative stimulus caused inhibition of cell division in the M phase through p38 MAPK activation. The kinase cascade may serve as a primary transducer of cytoplasmic oxidative signals to nucleus for stress-relieving gene expression and cell cycle control before apoptosis-inducing signals are transduced. This is the first report demonstrating that oxidative stress can participate in cell cycle control by induction of a signal cascade.

Experimental Procedures

Cell Culture and H2O2 Treatment—U937 cells were maintained with RPMI 1640 containing 10% fetal bovine serum and antibiotics in an atmosphere with 5% CO2. Before the H2O2 treatment, cells (5 × 106) were equilibrated with fresh medium (5 ml) consisting of RPMI 1640 and 2% serum for 2 h. After adding H2O2 to make 0.02 mM or 0.2 mM, cells were harvested at time intervals and washed in a buffer (0.15 mM NaCl, 10 mM Tris-HCl, pH 7.4, 2 mM EDTA, and 2 mM EGTA). For the experiment with NAC or SB203580, the compounds were added to the culture 1 h before the cells were exposed to H2O2. SB203580 was dissolved in dimethyl sulfoxide at 10 mM and diluted with the medium to 10 μM. Nocodazole stock solution (5 mM) in dimethyl sulfoxide was applied to the culture at a concentration of 5 μM.

Western Blot— Washed cells (107) were lysed in the sample buffer (100 μl) for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immediately boiled for 2 min. DNA was sheared by sonication. A 10-μl aliquot was loaded in each slot. Biotinylated protein standards (Bio-Rad) were used as size markers. After SDS-polyacrylamide gel electrophoresis, proteins were transferred to polyvinylidene difluoride membrane by electrophoresis. Membrane was blocked with 5% nonfat dry milk in TBST (0.15 mM NaCl, 10 mM Tris-HCl, and 0.1% Tween 20 at pH 7.4) and incubated with an antibody at a concentration of 100–200 ng/ml IgG in the blocking buffer. For detection of the phosphorylation status of p38 MAPK, MKK3/6, MKK4, JNK, c-Jun, or MKK4, an antiserum reactive with the phosphorylated form of each protein (phosphospecific antibody) was purchased from Bio-Rad. Antibodies of the 1E6-α, NF-κB p65, and p38 MAPK protein composition were performed with antisera from Santa Cruz (1E6-α—C2), NF-κB (A), and p38 MAPK (H-147)). The membrane was washed in TBST with brief sonication. By using alkaline phosphatase-conjugated secondary antibodies and the CDP-star chemiluminescent system (Bio-Rad), protein bands were visualized.

Nuclear Staining—Cells were fixed in glutaraldehyde (1.25% in phosphate-buffered saline) for 16 h at 4 °C, and then the glutaraldehyde was washed away with phosphate-buffered saline. Cell suspension was combined with Hoechst 33342 solution (6 mM) at 5:1 and observed under fluorescence microscopy.

Flow Cytometry—Cells fixed in 70% ethanol were stained with propidium iodide (PI) (50 μg/ml) after RNA digestion. PI-stained 10,000 cells were analyzed for DNA content with a Becton Dickinson FACScan flow cytometer.
Oxidative Stress Causes Mitotic Arrest

RESULTS

Analysis of the Kinase Cascades Responsive to Oxidative Stresses—U937 cell lysates prepared after 0-, 10-, 30-, 60-, and 120-min incubation with 0.02 or 0.2 mM H$_2$O$_2$ were examined for activation of p38 MAPK, JNK, and IxB kinase signal transduction pathways by Western blotting. p38 MAPK activation was analyzed with an antisera reactive with the protein doubly phosphorylated on Thr-183 and Tyr-185 (P-p38 MAPK). JNK and IxB kinase signal transduction pathways by Western blotting. p38 MAPK phosphorylation was substantially enhanced (Fig. 1a, i) without alteration of the p38 MAPK protein amount (Fig. 1b). The phosphorylation was retained at least for 120 min. NAC, an anti-oxidant activated in a cytoplasmic redox enzyme system (8), completely blocked it (Fig. 1c), indicating that an intracellular oxidative stimulus was generated to induce p38 MAPK. The moderate, temporary phosphorylation of CREB (ATF-1) may reflect the activity of mitogen-activated protein kinase-activated protein kinase-2, a cytoplasmic substrate of p38 MAPK (20). The strong induction of CREB (ATF-1) confirms that the oxidative stimulus caused activation time course observed in Fig. 1. However, c-Jun phosphorylation appeared at 10 min (Fig. 2g). This induction was blocked either by NAC (Fig. 2d) or by 10 μM p38 MAPK-specific inhibitor SB203580 (Fig. 2e), indicating that the oxidative signal was immediately relayed to nuclear factor ATF-2 by p38 MAPK activation. On the other hand, Ser-73 phosphorylation of NF-κB p65 in the nuclear fractions (Fig. 1f), indicating occurrence of NF-κB nuclear translocation through IκB degradation (17). Thus, cells responded to the stress of 0.02 mM H$_2$O$_2$ by activation of p38 MAPK, without affecting either JNK or NF-κB cascade. In contrast, all of the three stress-responsive protein kinase cascades were activated by the 0.2 mM H$_2$O$_2$ treatment.

Fig. 1. Analysis of the stress signal cascades under the two different levels of oxidative stress. Cells treated with 0.02 mM (a–h) or 0.2 mM H$_2$O$_2$ (i–n) for the indicated periods were examined for the phosphorylation status of kinases and for protein composition by Western blotting. Proteins detected are as follows: a and i, p38 MAPK with a double phosphorylation on Thr-180 and Tyr-182 (P-p38 MAPK); b, p38 MAPK total protein; c, p-p38 MAPK in the presence of NAC; d and j, JNK1 and JNK2 doubly phosphorylated on Thr-183 and Tyr-185 (P-JNK1 and P-JNK2, respectively); e and k, IκB-α protein; f and l, NF-κB p65 protein in nuclear fraction; g and m, MKK4 with Thr-223 phosphorylation (P-MKK4); h and n, MKK3 and MKK6 with a double phosphorylation of Ser-189/Thr-193 and Ser-207/Thr-211, respectively (P-MKK3/6). Positions of size markers (molecular masses indicated in kDa) are marked with bars on the left of panels a–h. In i–n, sizes are as shown to the left of panels a–h.

Fig. 2. Phosphorylation of transcription factors under the low level oxidative stress. Concentrations of H$_2$O$_2$ and compound included in the culture are indicated to the left of each panel. Lanes correspond to the time course (in min) shown above. Proteins detected are as follows: a and b, CREB and ATF-1 with phosphorylation on Ser-133 (P-CREB and P-ATF1, respectively); c–e, ATF2 with Ser-73 phosphorylation (P-ATF2), and f and g, c-Jun with Ser-32 phosphorylation (P-cJun).
0.02 mM H₂O₂-treated cells (Fig. 3a). DNA staining with Hoechst 33342 dye of the cells fixed at 24 h revealed the presence of deformed, polyploid nuclei in 38.5 ± 7.8% (n = 200) cells. Flow cytometric analysis with PI staining showed an increased cell count in the fractions of 4N DNA content (M5 plus M6, 10.9%) in comparison with the control culture (Fig. 3f), which had a 2.3% population in the M5/M6 fractions. The ratio of cells with 4N DNA to cells with 2N DNA increased more than 2-fold (0.87) comparing with that in the control culture (0.39). Thus, U937 cells treated with 0.02 mM H₂O₂ underwent an abnormal cell cycle progression in which S phase entry occurred without completion of the M phase events, the phenomenon referred to as “S/M uncoupling” (21).

When cultured with 5 ng/ml nocodazole, a compound that blocks tubulin assembly, the level of p38 MAPK phosphorylation gradually rose (Fig. 3b) as reported (14). Hoechst 33342 staining revealed an abundance of enlarged nuclei in the nocodazole-treated culture at 24 h. The ratio of cells with ≥4 N DNA to cells with 2 N DNA increased more than 2-fold (0.87) comparing with that in the control culture (0.39). Thus, U937 cells treated with 0.02 mM H₂O₂ underwent an abnormal cell cycle progression in which S phase entry occurred without completion of the M phase events, the phenomenon referred to as “S/M uncoupling” (21).

As demonstrated by an enzymological analysis (22), SB203580 blocked not only ATF-2 phosphorylation (cf. Fig. 2e) but also the p38 MAPK phosphorylation itself under the stress of 0.02 mM H₂O₂ (Fig. 3c). The p38 kinase inhibition continued for 24 h and abolished polyploid cell formation as found by flow cytometry. Moreover, nuclear fragmentation was observed in 8.73% of cells after the 24-h culture with the inhibitor and 0.02 mM H₂O₂. The blockage of p38 MAPK cascade might have interfered with an anti-oxidative cellular defense mechanism, which led to the onset of apoptosis in the small fraction of the culture.

NAC completely canceled both p38 MAPK cascade activation and polyploid cell formation (Fig. 3d) without inducing apoptosis. Furthermore, in the experiment where 0.02 mM H₂O₂ was washed away from the culture after the first 2-h incubation period, the level of p38 MAPK phosphorylation fell to the basal level by 3 h from the H₂O₂ removal. Those cells, examined at the end of the total incubation period of 24 h, were indistinguishable from the control cells either by flow cytometry or Hoechst staining (Fig. 3, e and f). Thus, the kinase activation was found to be a reversible response, and its prolonged activation was required for polyploid cell formation. Under the high level oxidative stress conditions caused by 0.2 mM H₂O₂, occurrence of nuclear fragmentation was detectable as early as at 5 h. Approximately 95% of cells displayed features of apoptosis including nuclear fragmentation and membrane blebbing (Fig. 3g).

**DISCUSSION**

Although other various signal pathways have been left unexamined, it is evident that p38 MAPK is activated selectively among the stress-responsive signal cascades under the low level oxidative stress conditions with 0.02 mM H₂O₂. Furthermore, the result with p38-specific inhibitor SB203580 suggests that p38 MAPK bears an important function for cell survival. Oxidative stresses at different levels seem to join different streams of the cellular signal transduction system reaching to...
specific nuclear factors (23), although the mechanism remains to be determined. MKK3/6 may at least in part contribute to the selective p38 activation under the low level oxidative stress. MKK4 (SEK1)-JNK activation coincides with IkB degradation in response to 0.2 mM H$_2$O$_2$, suggesting that mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1 (24, 25) is possibly involved in transmission of the high level, apoptosis-inducible oxidative stimulus to the IkB kinase and JNK pathways. Given that the normal function of mitochondria inevitably produces ROS, the results in this study permit a speculation that p38 MAPK serves as a signal transducer of the cytoplasmic low level ROS for balancing the transduction of genes harboring their binding sites in the promoter region, such as the manganese superoxide dismutase gene (26).

This study provides a biological interpretation for the mechanistically demonstrated M phase arrest dependent on p38 MAPK activation in Xenopus embryos and NIH3T3 cells (14). When a low level oxidative stress occurs in cytoplasm, it could induce the cellular reactions. Unlike those cells, however, U937 cells lack functional p53 (27), which controls the spindle assembly checkpoint (28) as well as the G$_2$/S transition. It may explain why S/M uncoupling rather than M phase cell cycle arrest was observed in U937 cells. The p38 kinase activation was found reversible, suggesting that it could function as a sensitive, flexible mediator of oxidative signals. Cells possibly gain time by cell cycle retardation to escape from the oxidative conditions before the stress grows to cause a serious damage in chromosome or cell functions.

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