p38 Mitogen-activated Protein Kinase Mediates Hypoxic Regulation of Mdm2 and p53 in Neurons*

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The multifunctional tumor suppressor protein, p53, inhibits cell growth and promotes differentiation and programmed cell death. p53 activity is controlled by transcriptional, translational, and post-translational regulation. A major pathway for post-translational regulation of p53 comprises its nucleocytoplasmic transport and subsequent proteasomal degradation, which involves binding to the oncoprotein, murine double minute-2 (Mdm2). Hypoxia and other stress signals cause cellular injury partly through the action of p53. In this study, we show that hypoxia induces down-regulation of Mdm2 as well as serine 15 phosphorylation and nuclear accumulation of p53 in cultured cortical neurons from E16 mice. These effects are diminished by the p38 mitogen-activated protein kinase inhibitors SB203580 and SB202474, and by a dominant-interfering mutant of the p38-activating kinase mitogen-activated protein kinase kinase 3 (MKK3). Hypoxic neuronal death was also reduced by p38 inhibitors, by dominant-interfering MKK3, and by a p53-antisense oligodeoxynucleotide and was increased by a constitutively active form of p38 and by an Mdm2-antisense oligodeoxynucleotide. These results demonstrate that p38 and Mdm2 have roles in coupling hypoxic-ischemic neuronal insults to activation of p53 and hypoxic cell death.

Hypoxia is an important pathophysiological feature of ischemic disorders, including stroke. Like other stress signals, neuronal hypoxia and ischemia cause DNA damage and cell death partly by promoting nuclear accumulation of the p53 tumor suppressor protein (1, 2). p53 is a multifunctional protein that has a critical role in various pathways controlling cellular responses to stress signals (for reviews, see Refs. 3 and 4). p53 is normally expressed at low levels, in a latent form that is unable to bind specifically to DNA, by rapid degradation through ubiquitin-dependent proteolysis (5). With stress, p53 accumulates via multiple mechanisms, including enhanced translation, decreased proteolytic degradation, and post-translational modification (6–8). Under these conditions, its half-life is extended from 30 to >200 min, contributing to an increase in p53 protein levels (7).

The murine double minute-2 (Mdm2) oncprotein, a product of a p53-responsive gene, is a major inhibitor of p53 function and abundance (9, 10). Mdm2 was originally identified as an amplified gene in a spontaneously transformed derivative of the BALB/c cell line, 3T3 DM (9). The Mdm2 gene contains a p53 DNA-binding site and a genetically responsive element such that expression of Mdm2 can be regulated by the level of wild-type p53 protein. The Mdm2 protein, in turn, can complex with p53 and decrease its ability to act as a positive transcription factor (10). In addition to its ability to antagonize p53-dependent transcription, Mdm2 can also promote degradation of p53 through a ubiquitin-dependent proteasome pathway (6, 11) and may have an additional role in nucleocytoplasmic shuttling of p53. This establishes a negative feedback loop, in which p53 initiates its own Mdm2-mediated destruction (10, 12). Post-translational modifications of p53 include phosphorylation and acetylation (8, 13), and phosphorylation of p53 at serine 15 and serine 20 leads to reduced binding to Mdm2, which enhances p53 accumulation (8, 14). Finally, reduced expression of Mdm2 has been implicated in the induction of p53 by hypoxia in non-neuronal cells (15).

How neuronal hypoxia or ischemia regulates p53 is unknown, but hypoxia influences a variety of signal transduction mechanisms, including mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt pathways (16–18). Among the MAPKs, extracellular signal-related kinase (ERK) has been widely associated with cell survival, whereas c-Jun amino-terminal kinases (JNK) and p38 are often implicated in cell death (16, 19). However, the actual roles of each MAPK cascade are highly cell type- and context-dependent (16). In the mouse JB6 epidermal cell line, activated ERK and p38, but not JNK, phosphorylates p53 at serine 15 and reduces binding of p53 to Mdm2 (8), whereas in mouse fibroblasts, JNK associates with p53 and promotes its ubiquitination (20).

Stress signals can also up-regulate p38 in neurons (19, 21, 22). For example, NO donors activate p38 and induce apoptosis in cortical neurons without activating JNK, and the p38 MAPK activity inhibitor SB203580 protects cortical neurons from NO-induced cell death (22). These and related findings prompted us to assess the relationship between p38 activity and levels of Mdm2 and p53 in primary cultures of cortical neurons subjected to hypoxic insults. We report here evidence that suggests a role for p38 MAPK in regulating Mdm2 and p53 levels in hypoxic neurons.
Fig. 1. Time course of Mdm2 and p53 mRNA expression in response to hypoxia. Cortical neurons were harvested after the indicated periods (h) of hypoxia. RNA samples (15 μg) were loaded on 1% agarose gels and transferred to Hybond-N membranes for Northern analysis. Blots were probed for Mdm2 or p53 and exposed on Fuji films, then re-probed for β-actin mRNA expression to control for variations in gel loading. Data shown are representative blots from three independent experiments per panel (A) and computer-assisted densitometric measurements (mean ± S.E., n = 3) (B). *, p < 0.05 relative to time 0 h.

EXPERIMENTAL PROCEDURES

Reagents—The MAPK kinase (MEK) inhibitor PD98059, the p38 inhibitors SB203580 and SB202474, the PI3K inhibitor wortmannin, the protein kinase C (PKC) inhibitor GF109203X, the tyrosine kinase receptor inhibitor genistein, and the Src family kinase inhibitor herbimycin A were from Calbiochem. Mouse anti-Mdm2 antibody and rabbit anti-p38 antibody were from Santa Cruz. Rabbit anti-p53, polyclonal anti-phospho(Ser15)-p53, polyclonal anti-phospho(Thr180/Tyr182)-p38 MAPK, and anti-phospho-SAPK/JNK antibodies were from Cell Signaling Technology, Inc. Rabbit anti-ERK 1/2 polyclonal antibody was from Promega. Mouse anti-β-actin monoclonal antibody was from Sigma. Media and sera were obtained from Invitrogen.

Cell Culture—Primary neuronal cultures were established as previously described (23, 24). In brief, cerebral cortex was dissected from fetal CD1 mice at 16 days of gestation, treated with trypsin for 3 min at 37 °C, and dissociated by trituration. Dissociated cell suspensions were plated at 3.5 × 10⁴ cells/cm² on plastic tissue culture dishes coated with poly-D-lysine, in defined medium (Neurobasal/B27, Invitrogen) supplemented with 2 mM glutamine, penicillin (25 units/ml), and streptomycin (25 μg/ml). Cultures were maintained in a humidified 5% CO₂ incubator at 37 °C for 5 days before treatment. Under these conditions, cultures contained ~95% neurons as reported previously (25).

Hypoxia—To induce hypoxia, cells were placed in modular incubator chambers (Billups-Rothenberg, Del Mar, CA) for 0–24 h at 37 °C in humidified 85% N₂, 5% CO₂ (25). To evaluate the effect of signaling pathways on Mdm2 and p53 expression, the inhibitors listed above were added 1 h before the onset of hypoxia.

Northern Blots—Northern blotting was carried out as described previously (26). Briefly, total RNA from treated and untreated cells was extracted using RNeasy Mini Kits (Qiagen), according to the manufacturer’s instructions. Fifteen micrograms of total RNA from each sample was fractionated on 1% formaldehyde/agarose gels and transferred to Hybond-N nylon membranes (Amersham Biosciences). Filters were hybridized with probes for Mdm2 mRNA, p53 mRNA, and β-actin mRNA at 68 °C in hybridization buffer (CLONTECH). 32P-Radiolabeled DNA probes were synthesized using cDNA obtained from reverse transcriptase-PCR amplification. Primers used were: p53 forward, 5'-ATGGAGAAGTCAAGATTGCTGAGTAATA-3'; p53 reverse, 5'-GACCTTCGGTAGATGGCCATGG-3'; Mdm2 forward, 5'-TCACGCAAGACTCTGGCACAATC-3'; Mdm2 reverse, 5'-TTCCGAGTCAGACATGCTGCTGGA-3'; β-actin forward, 5'-CACAGGGATTTGTGATGGACTCT-3'; β-actin reverse, 5'-GCTCAGGGAGGAAATGACT-3'.

Western Blots—Cells were washed twice in phosphate-buffered saline, and whole cell extracts were prepared by adding 10 volumes of 1 × sample buffer (2% SDS, 100 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 0.5 μg/ml benzamidine). The cell suspension was incubated on ice for 15 min before adding Nonidet P-40 to a final concentration of 0.6%, vortexed, and centrifuged at 13,000 g for 15 min. Nuclear extracts were performed as previously described (27) with modifications. Briefly, cells were washed with cold phosphate-buffered saline, collected by centrifugation at 1000 × g for 5 min, and then resuspended in buffer A (10 mM Heps, pH 7.9, 10 mM KCl, 0.1 mM EDTA, pH 8.0, 0.1 mM EGTA, pH 8.0, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 0.5 μg/ml benzamidine). The cell suspension was incubated on ice for 15 min before adding Nonidet P-40 to a final concentration of 6.0%, vortexed, and centrifuged at 13,000 × g for 15 min. The supernatant was collected as the cytoplasmic fraction, and the nuclear pellet was washed with buffer A (without Nonidet P-40) and suspended in buffer B (20 mM Heps, pH 7.9, 400 mM NaCl, 1 mM EDTA, pH 8.0, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 0.5 μg/ml benzamidine). After incubation on ice for 15 min, the suspension was centrifuged at 13,000 × g for 15 min. The nuclear extract was collected, and protein concentration was determined using Bradford protein assays (Bio-Rad); 40 μg of protein was analyzed by 10 or 12% SDS-PAGE and transferred to Immobilon PVDF membrane (Bio-Rad). Mem-
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**RESULTS**

**Hypoxia Down-regulates Mdm2 and Up-regulates p53 in Cortical Neurons**—To determine whether hypoxia alters the expression of p53 and Mdm2 genes in neurons cultured from mouse cerebral cortex, cells were deprived of oxygen for 0–24 h, total RNA was extracted, and p53 and Mdm2 expression was analyzed by Northern blotting. Mdm2 was prominently down-regulated in a time-dependent manner, while p53 increased, compared with the control gene, β-actin (Fig. 1). Western analysis of Mdm2 and p53 proteins in nuclei and cytoplasm of cells fractionated after 0–24 h of hypoxia showed a reduction in Mdm2 protein levels in both compartments and an increase in nuclear p53 (Fig. 2A). Each of these changes was detectable after 4 h and persisted for at least 24 h. At all times examined, Mdm2 expression was most pronounced in the cytoplasm, and p53 predominated in the nucleus.

**Hypoxia Induces Phosphorylation of p53 at Serine 15**—Phosphorylation of p53 at serine 15 helps to regulate p53 stability by reducing its interaction with Mdm2 and has been reported after UV exposure (8). To explore the possibility that p53 is also phosphorylated in response to hypoxia, we analyzed cell extracts from normoxic and hypoxic cortical neurons by Western blotting with an antibody directed against serine 15 phospho-p53. As shown in Fig. 2B, the level of serine 15 phospho-p53

**Cell Viability Assay**—Neuronal cell viability was assessed by measuring formazan produced by the reduction of 3-(4,5-dimethylthiazol-2-

**Fig. 3.** Hypoxia-induced phosphoactivation of ERK and p38 but not JNK in cortical neurons. Cortical neurons were harvested after the indicated periods (h) of hypoxia. Protein samples (150 μg) from whole cell extracts were loaded on 12% SDS-PAGE gels and transferred to PVDF membranes for Western analysis. Phospho-ERK1/2, phospho-p38, phospho-JNK, total ERK1/2, and total p38 from whole cell extracts were detected with the anti-Thr182/Tyr185 phospho-ERK1/2, anti-Thr180/Tyr182 phospho-p38, anti-phospho-JNK, anti-ERK1/2, and anti-p38 antibodies described under “Experimental Procedures.” Data above are representative blots from three independent experiments per panel.

**Fig. 4.** Effects of protein kinase inhibitors on hypoxia-induced down-regulation of Mdm2, accumulation of p53, and phosphorylation of p53 at serine 15. Cortical neurons were treated with the following inhibitors for 1 h prior to the onset of 16 h of hypoxia: herbimycin A (1 μM), genistein (50 μM), SB203580 (5 μM), GF109203X (1 μM), wortmannin (2 μM), and PD98059 (20 μM). Protein samples (40 μg) from whole cell extracts were loaded on 10% SDS-PAGE gels and transferred to PVDF membranes for Western analysis. Mdm2, p53, Ser15 phospho-p53, and β-actin in whole cell extracts were detected with the anti-Mdm2, anti-p53, anti-Ser15 phospho-p53, and anti-β-actin antibodies described under “Experimental Procedures.” Data shown are representative blots from three independent experiments per panel.
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Although it became detectable when we used SB202474 (4 μM) or SB202190 (4 μM) for 1 h prior to the onset of 16 h of hypoxia (A) or normoxia (B). Protein samples (40 μg) from whole cell extracts were loaded on 10% SDS-PAGE gels and transferred to PVDF membranes for Western analysis. Mdm2 and p53 from total cell extracts were detected with the anti-Mdm2 and anti-p53 antibodies described under “Experimental Procedures.” Data shown are representative blots from three independent experiments per panel.

Increased as the duration of hypoxia increased up to 16 h and then declined at 24 h, perhaps due to hypoxic cell death. In contrast to the effect of hypoxia on serine 15 phosphorylation, no change in serine 20 phosphorylation of p53 could be detected (not shown).

Hypoxia Activates ERK and p38 MAPK but Decreases JNK Activity—The specific signal transduction pathways involved in cellular responses to hypoxia vary with cell type; for example, HeLa cells respond with a rapid but transient activation of ERK (17), whereas p38 (but not JNK) is selectively activated in PC12 cells (31). To understand the mechanisms underlying the down-regulation of Mdm2 and serine 15 phosphorylation and up-regulation of p53 in hypoxic cortical neurons, we measured changes in the expression of phosphoactivated forms of ERK, p38, and JNK. Using phosphospecific antibodies against ERK, p38, and JNK, we found that phospho-ERK1 and phospho-p38 were present at low levels in normoxic neuronal cultures and that exposure to hypoxia for 4 to 24 h caused a progressive increase in immunoreactivity; phospho-p38 was also detected as early as 4 h after hypoxia and remained elevated for at least 24 h (Fig. 3). In contrast, antibodies against total p38 or total ERK1/2 showed no hypoxia-induced change in expression. Thus, both ERK and p38 were phosphoactivated by hypoxia in cortical neurons, whereas hypoxia suppressed phosphoactivation of JNK. In this study, the level of p38 in cortical neurons appeared to be much lower than that of the other two MAPKs, although it became detectable when we used >150 μg of protein per lane.

Inhibition of p38 Blocks Hypoxic Down-regulation of Mdm2, as Well as Serine 15 Phosphorylation and Accumulation of p53—To investigate further which signaling pathways mediate the effects of hypoxia on Mdm2 and p53 in neurons, we used a series of kinase inhibitors to inactivate receptor tyrosine kinases, ERK, p38, JNK, P13K, and PKC. We expected that if a particular kinase were critical for hypoxia’s effects on Mdm2 and p53 signaling, inhibiting its activity should reverse these effects. As shown in Fig. 4, the Src family kinase inhibitor herbimycin A (1 μM) (32) increased Mdm2 expression in hypoxic cultures, and the p38 inhibitor SB203580 (5 μM) (33) prevented hypoxic down-regulation of Mdm2, whereas the non-selective tyrosine kinase inhibitor genistein (50 μM) (32) was ineffective, and the PKC inhibitor GF102390X (1 μM) (33), the P13K inhibitor wortmannin (2 μM) (18), and the ERK inhibitor PD98059 (20 μM) (33) potentiated the effect of hypoxia on Mdm2. Genistein and SB203580 also blocked hypoxic up-regulation of p53, whereas the other inhibitors were less effective or inactive in this regard. As is also shown in Fig. 4, SB203580 and wortmannin markedly reduced hypoxia-stimulated phosphorylation of p53 on serine 15. Because only the p38 inhibitor SB203580 reversed all of the observed effects of hypoxia, down-regulation of Mdm2, up-regulation of p53, and serine 15 phosphorylation of p53, hypoxic regulation of Mdm2/p53 signaling appears to be mediated through p38. To verify further that this is the case and to control for nonspecific effects, we treated cells with another p38 inhibitor (SB202190, 4 μM) or with SB202474 (4 μM), an inactive analog of SB203580 and SB202190. Western blots showed that the effects of SB202190 and SB203580 on Mdm2 and p53 expression were similar, whereas SB202474 was ineffective (Fig. 5A). The p38 inhibitors appeared to act by blocking hypoxia-induced changes in Mdm2 and p53 expression specifically because neither inhibitor altered expression under normoxic conditions (Fig. 5B).

p38 Activity Is Required for Hypoxia-induced Cell Death—To investigate the relationship between hypoxic induction of p38 and hypoxia-induced cell death, we first treated cells with the p38 inhibitor SB203580 (5 or 10 μM) (SB) for 1 h and then exposed to hypoxia for 8 or 16 h. Cell viability was measured by MTT absorbance and expressed as a percentage (mean ± S.E., n = 3) of viability in normoxic cultures treated with Me2SO vehicle (Control). *, p < 0.05 relative to control.
Knockdown of Mdm2 in Cortical Neurons Reduces Cell Viability and Elevates p53 Expression—To evaluate the functional consequences of hypoxic reduction of Mdm2 expression, we blocked Mdm2 expression with an antisense ODN (MAS20), which was transfected into cells for 24–48 h as described previously (36); a scrambled ODN (MX20) was used as a control. As shown in Fig. 8A, blocking Mdm2 expression with MAS20 reduced cell viability by ~40% under normoxic conditions and by ~35% after hypoxia. Western blots showed that knockdown of Mdm2 by MAS20 reduced Mdm2 and increased p53 levels, as expected (Fig. 8B).

Knockdown of p53 Rescues Cortical Neurons from Hypoxic Cell Death—To assess if p53 is required for hypoxic neuronal cell death, we transfected cells with a p53 antisense ODN that overlaps the translation start site. The p53 antisense ODN did not affect cell viability under normoxic conditions, relative to cells transfected with a sense ODN or treated with transfection reagent only. In contrast, in hypoxic cultures, the p53 antisense ODN increased neuronal viability from ~40 to ~70% (Fig. 9). Because basal p53 expression was barely detectable, it was not possible to discern a further decrease in expression after antisense treatment.

**DISCUSSION**

The major finding of this study is that neuronal hypoxia transcriptionally down-regulates Mdm2 and post-transcriptionally up-regulates p53 and that both effects may be mediated through hypoxic activation of p38 MAPK. These events appear to have functional importance in regulating neuronal cell death and survival from hypoxia, because hypoxic neuronal death was reduced by p38 inhibitors, by a dominant-interfering mutant of the p38-activating kinase MKK3, and by a p53-antisense ODN and was increased by a constitutively active form of p38 and by an Mdm2-antisense ODN.

The known and proposed roles of Mdm2 in regulating p53 function suggest that the effects of hypoxia on Mdm2 and p53 may be interrelated (37). First, because binding to Mdm2 promotes the proteasomal degradation of p53 through Mdm2’s action as a ubiquitin ligase (36), reduction of Mdm2 expression by hypoxia could account for the observed increase in p53 levels in our hypoxic neuronal cultures. This is supported by our finding that an Mdm2-antisense ODN increased p53 abundance. Second, the observed increase in serine 15 phosphorylation of p53 would also be expected to increase p53 levels because this post-translational modification interferes with the interaction between p53 and Mdm2 that leads to p53 degradation (8). Finally, some evidence suggests that Mdm2 may be involved in the nucleocytoplasmic shuttling of p53 that translocates it from its site of action as a transcription factor to its...
through several signal transduction pathways. We found evidence that viability argues for a survival-promoting effect of Mdm2 in this system. Reduced Mdm2 expression promotes either cell death or cell survival, depending on the stimulus. In the present study, the observation that an Mdm2-antisense ODN reduced neuronal viability argues for a survival-promoting effect of Mdm2 in this system.

FIG. 9. Antisense knockdown of p53 rescues neurons from hypoxic cell death. Cortical neurons were transfected for 24 h with 5 μM p53 antisense (p53AS) or p53 sense (p53S) ODN as described under “Experimental Procedures” and then exposed to hypoxia or normoxia for an additional 16 h. A, cell viability was measured by MTT absorbance and expressed as a percentage (mean ± S.E., n = 5) of viability in untransfected cultures (No ODN). *, p < 0.05 relative to p53S. B, protein samples (40 μg) from whole cell extracts were loaded on 10% SDS-PAGE gels and transferred to PVDF membranes for Western analysis, and Mdm2 and p53 were detected with the antibodies described under “Experimental Procedures.” Data shown are representative blots from three independent experiments per panel.

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