Early Single Cell Bifurcation of Pro- and Antiapoptotic States during Oxidative Stress*[S]

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In a population of cells undergoing oxidative stress, an individual cell either succumbs to apoptotic cell death or maintains homeostasis and survives. Exposure of PC-12-D2R cells to 200 μM hydrogen peroxide (H₂O₂) induces apoptosis in about half of cells after 24 h. After 1-h exposure to 200 μM H₂O₂, both antiapoptotic extracellular regulated kinase (ERK) phosphorylation and pro-apoptotic Ser-15-p53 phosphorylation are observed. Microarray and real-time PCR assays of gene expression after H₂O₂ exposure identified several transcripts, including egr1, that are rapidly induced downstream of ERK. Single cell analysis of egr1 induction and of phospho-ERK and phospho-p53 formation revealed the presence of two distinct cellular programs. Whereas the proportion of cells activating ERK versus p53 at 1 h depended on H₂O₂ concentration, individual cells showed exclusively either phospho-p53 formation or activation of ERK and egr1 induction. Exposure to H₂O₂ for 1 h also elicited these two non-overlapping cellular responses in both dopaminergic SN4741 cells and differentiated postmitotic PC-12-D2R cells. Repressing p53 with pifithrin-α or small interfering RNA increased ERK phosphorylation by H₂O₂, indicating that p53-dependent suppression of ERK activity may contribute to the bi-stable single cell responses observed. By 24 h, the subset of cells in which ERK activity was suppressed exhibit caspase 3 activation and the nuclear condensation characteristic of apoptosis. These studies suggest that the individual cell rapidly and stochastically processes the oxidative stress stimulus, leading to an all-or-none cytoprotective or pro-apoptotic signaling response.

Reactive oxygen species (ROS)† have been implicated in the pathophysiology of several human diseases, including atherosclerosis, pulmonary fibrosis, cancer, neurodegenerative disorders, and aging (1–3). Although the cytotoxic actions of ROS are well known, ROS are increasingly recognized as components of cellular signaling that modulate responses in both physiological and pathological conditions (4). For example, ROS are produced in muscle cells upon binding of ligands such as angiotensin II (5). In addition, ROS production has been documented in a number of cells stimulated with cytokines, including tumor necrosis factor-α, transforming growth factor-β, and interleukin-1 (6–8), and with such growth factors as bovine fibroblast growth factor, nerve growth factor, platelet-derived growth factor, and epidermal growth factor (9–11). These observations suggest that ROS can damage various cell components or activate specific physiological signaling pathways, with the relative effects determined by ROS concentration.

Oxidative stress has been reported to activate seemingly contradictory signaling pathways, and the consequences of the response vary widely; the ultimate outcome is dependent on the balance between these stress-activated pathways (12, 13). Among the main stress signaling pathways and/or central mediators activated in response to oxidant injury are the extracellular regulated kinase (ERK) (14–16), c-jun amino-terminal kinase (JNK) (17–19), p38 mitogen-activated protein kinase (20) signaling cascades, the phosphoinositide 3-kinase/Akt pathway (21), the nuclear factor-κB signaling system (22, 23), p53 mitogen-activated protein kinase (24, 25), and the heat shock response (26). In general, the heat shock response, ERK, phosphoinositide 3-kinase/Akt, and nuclear factor-κB signaling pathways exert a pro-survival influence during oxidant injury, whereas activation of p53, JNK, and p38 are implicated in apoptosis (see review in Ref. 12).

ROS, including hydrogen peroxide (H₂O₂), are natural by-products generated by living organisms as a consequence of aerobic metabolism (27). The cellular toxicity of H₂O₂ is associated with the rapid modification of cellular constituents, including the depletion of intracellular glutathione and ATP, a decrease in NAD⁺ level, an increase in free cytosolic Ca²⁺, and lipid peroxidation (28). H₂O₂ also activates the opening of the mitochondrial permeability transition pore and the release of cytochrome c (29). In the cytoplasm, cytochrome c, in combination with Apaf-1, activates caspase-9 leading to the activation of caspase-3 and subsequent apoptosis (30). The initiating events leading to activation of these different signaling pathways in response to H₂O₂ are incompletely understood.

We have reported recently that H₂O₂ induces apoptosis in PC-12-D₂R cells and in the nigral dopaminergic neuronal cell line SN4741 in a concentration-dependent manner (31, 32). These observations suggest that when exposed to a level of oxidative stress that can induce apoptosis in a portion of cells, each individual cell must proceed through a decision-making process that ultimately results either in its survival or its death. We report here that early after H₂O₂ exposure, each cell activates either homeostatic or proapoptotic signaling pathways, but not both. Our results indicate that it may be chal-

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[S] The on-line version of this article (available at http://www.jbc.org) contains a supplemental table.

†The abbreviations used are: ROS, reactive oxygen species; ERK, extracellular regulated kinase; JNK, c-jun N-terminal kinase; qPCR, quantitative real time PCR; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; FISH, fluorescent in situ hybridization; siRNA, small interfering RNA.
Reactive Oxygen Species Activated Signaling Pathways

lenging to develop models of the mechanism of oxidative stress-induced cell death based solely on cell population and biochemical assays.

EXPERIMENTAL PROCEDURES

Materials—U34 oligonucleotide array gene chips were from Affymetrix (Santa Clara, CA) and total RNA isolation kit was from Stratagene (La Jolla, CA). Antibodies specific to phospho-ERK, ERK, phospho-p53, p53, phospho-JNK, JNK, phospho-p38 kinase, and p38 kinase were from Cell Signaling Technology (Beverly, MA). Anti-active caspase-3 antibody was from Promega (Madison, WI). Enhanced chemiluminescence lighting (ECL) Western blotting detection reagent kit was from Amersham Biosciences (Piscataway, NJ). Alexa fluor 488, goat anti-mouse alexa fluor 568, and goat anti-rabbit alexa fluor 488 conjugated secondary antibodies were from Molecular Probes (Eugene, OR). CY3 and atlas nucleosin columns were from BD Biosciences Clontech. Donkey anti-rabbit CY3 was from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Maxiscript in vitro transcription kit and aminothiol-UTP were from Ambion (Austin, TX). PD98059 and was from Calbiochem (San Diego, CA), and pïthrin-α was obtained from A.G. Scientific (La Jolla, CA). Qiaex II gel extraction kit and pDrive vector were from Qiagen (Valencia, CA). All PCR reagents were from Invitrogen.

Cell Culture—PC-12-D3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 500 μg/ml G418 (Invitrogen), 10% horse and 5% fetal bovine serum (Invitrogen) in a humidified atmosphere containing 5% CO2 at 37 °C. For differentiation, PC-12-D3 cells were plated on collagen-coated plates in Dulbecco's modified Eagle's medium containing 10% horse serum and 5% fetal bovine serum and allowed to attach overnight. The cells were then induced for differentiation by growing in Dulbecco's modified Eagle's medium supplemented with 0.5% fetal bovine serum and 100 ng/ml nerve growth factor for 7 days. Substantia nigra dopaminergic neuronal cell line SN4741 was cultured as described previously (33). Immunoblotting—Cells were washed twice with ice-cold phosphate-buffered saline and lysed in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Igepal C630, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 5 μg/ml aprotinin, and mixture of protease inhibitors (Roche Diagnostics GmbH) at 4 °C for 20 min. After centrifugation at 14,000 × g for 20 min at 4 °C, equal amounts of proteins were resolved by SDS-polyacrylamide gel electrophoresis. The proteins were electrotransferred to nitrocellulose membranes and detected by immunoblotting using the ECL system according to the manufacturer's recommendations. The blots were then stripped in buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol for 30 min at 50 °C and re-probed with respective antibodies.

Gene Expression Analysis—PC-12-D3 cells were treated with 200 μM H2O2 or vehicle for 1 h, and total RNA was isolated using StrataPrep total RNA miniprep kit according to the manufacturer's protocol. Preparation of cRNA, hybridization, and scanning of the rat genome U34 arrays were performed as described previously (34, 35). Affymetrix microarray suite 5.0 was used to analyze the raw data using the criteria of 60% concordance across multiple array comparisons and fold changes ≥1.6 for outlier detection. For each of the up-regulated targets, quantitative real-time polymerase chain reaction (qPCR) was carried out in an ABI Prism 7900HT (Applied Biosystems, Foster City, CA) using SYBR-Green assay, as described previously (35, 36). All the gene-specific primer sets used for qPCR are listed in supplemental table. The authenticity of the PCR products was verified by melting curve analysis and agarose gel electrophoresis. The comparative cycle threshold (Ct) method was used to analyze the data by generating relative values of the amount of target cDNA. Relative quantitation for any given gene, expressed as fold variation over control (untreated cells), was calculated after determination of the difference between Ct of the given gene A and that of the calibrator gene B (β-actin) in treated cells (ΔCtA) and untreated controls (ΔCtB), using the 2−ΔΔCt formula (37). Cq values are means of triplicate measurements. Experiments were repeated three to five times.

Immunocytochemistry—PC-12-D3 cells growing on collagen-coated or SN4741 cells growing on ploy-ornithine-coated cover glass were treated as indicated. The cells were fixed and permeabilized as described previously (31, 32), and immunocytochemical staining for phospho-ERK, phospho-p53, or active caspase-3 was carried out. Anti-phospho-ERK (1:400), anti-phospho-p53 (1:500), or active caspase-3 antibody (1:200) was added and incubated overnight at 4 °C. For double-immunolabeling, a mouse monoclonal anti-phospho-ERK antibody was used. After washing, cells were incubated with corresponding secondary antibodies for 2 h at room temperature. The cells were washed three times in phosphate-buffered saline, and the nuclei were stained with 1 μg/ml (in phosphate-buffered saline) of the fluorescent DNA dye 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) for 10 min and then washed with phosphate-buffered saline. The liquid was drained and the slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA) mounting medium.

Fluorescent in Situ Hybridization—Egr1 (161 bp) and β-actin (150 bp) DNA fragments were amplified from RNA isolated from PC-12-D3 cells by reverse transcription PCR using primer sets described in supplemental table. The DNA fragments were purified from agarose gel using QIAEX II gel extraction kit and sub-cloned into pDrive vector. Individual clones were sequenced to determine the orientation of the DNA fragments. We generated cRNA probes from Egr1 by nick translation using SP6 promoter for sense probes to minimize the interference of vector sequences in double fluorescent in situ hybridization (FISH). Amino-allyl-UTP incorporated cRNA probes were generated using Maxiscript in vitro transcription kit. The yield and integrity of riboprobes was confirmed by gel electrophoresis. The egr1 and β-actin cRNA probes were labeled with Alexa Fluor 488 and CY3, respectively, according to manufacturer’s protocols. The riboprobes were purified on atlas nucleosin columns. The cells grown on cover slips were fixed and permeabilized as described previously (31, 32). After prehybridization in 5× SSC, 50% formamide, and 1 mg/ml TRNA at room temperature for 30 min, the denatured probe was added to the prehybridization buffer. Hybridization was carried out for 2 h at 52 °C. After two 5-min washes in 5× SSC, 50% formamide, 0.1% SDS, and twice in 2× SSC, the nuclei were stained with DAPI, and slides were mounted in Vectashield mounting medium. When immunostaining was carried out after FISH, the slides were incubated with respective antibodies as described above.

RNA Interference—Custom SMARTpool plus small interfering RNA (siRNA) to target rat p53 (GenBank™ accession number NM_030989) was designed and synthesized by Dharmacon (Lafayette, CO). siRNA (50 pmol) was transfected into PC-12-D3 cells using transfect-TKO transfection reagent (Mirus, Madison, WI) as described previously (32). After 48 h of transfection, the cells were treated with H2O2 (200 μM) or vehicle for 1 h, and total RNA or cell extract was prepared. A nonspecific RNA duplex (Dharmacon) was used in control experiments.

RESULTS

ERK and p53 Pathways Are Activated in Response to Oxidative Stress—PC-12-D3 cells and substantia nigra dopaminergic SN4741 cells undergo apoptosis when exposed to H2O2 in a concentration- and time-dependent manner (31, 38). Oxidative stress is known to activate multiple signal transduction pathways in many experimental systems (12). To identify the signaling mechanisms activated by PC-12-D3 cells in response to H2O2, we have assessed the activation of ERK, JNK, p38 kinase, and p53, using Western blot analysis with antisera against phospho-ERK, phospho-JNK, phospho-p38 kinase, and phospho-p53. We found that 200 μM H2O2 rapidly induced the phosphorylation of ERK but not of JNK or p38 kinases in PC-12-D3 cells (Fig. 1A, B, and C). The activation of ERK by H2O2 was rapid and sustained (Fig. 1A, top). Anti-phospho-Ser15 antibody was used to detect oxidative stress-induced phosphorylation of p53, presumably caused by DNA damage (39–41) after exposure to H2O2 for periods up to 6 h. In PC-12-D3 cells, p53 phosphorylation was significantly enhanced within 30 min after H2O2 exposure and continued to increase for up to 2 h (Fig. 1D). The level of total p53 protein on Western blot analysis was unchanged after 6 h of incubation with H2O2 (Fig. 1D). These results demonstrate the early activation of ERK and p53 signaling pathways in response to oxidative stress in PC-12-D3 cells.

Characterization of ERK-activated Gene Program in Response to Oxidative Stress—ROS, through its effects on cell signaling, alters the expression of specific genes (42). To identify the genomic response during oxidative stress, the gene expression profile associated with H2O2 exposure was studied using oligonucleotide microarrays and regulated transcripts were confirmed by qPCR. As shown in Table I, genes that encode transcription factors including egr1, c-fos, jun, and p03,
and a zinc finger protein (copeb) were up-regulated after 200 μM H2O2 for 1 h. Other up-regulated genes include inner mitochondrial membrane component ATP synthase subunit c, stress response gene 70-kDa heat shock protein (hsp70), and the immediate-early inducible small GTP binding protein rhoB. We also found that the mitogen-activated protein kinase phosphatase-1 (mkp1) was increased by >3-fold in cells treated with H2O2. Several immediate early genes, such as egr1, c-fos, and c-jun, have been reported to be transcriptionally activated by increased cellular oxidation (16, 43, 44). Oxidative stress has also been shown to induce rhoB (45) and hsp70 (26).

To identify the component of the oxidative stress-induced gene program downstream of ERK activation, we inhibited ERK with PD98059. Addition of PD98059 (100 μM) 1 h before H2O2 treatment decreased the induction of egr1, pc3, and mkp1 (Fig. 2), genes that have been identified as downstream of ERK in other experimental systems (46–48). PD98059 did not prevent the induction of c-fos, copeb, c-jun, hsp70, rhoB, ATP synthase subunit c, and expressed sequence tag (GenBank accession number A639167).

Characterization of egr1 Induction in Response to Oxidative Stress—To investigate the cellular segregation of the diverse responses to oxidative stress, we next studied the induction of egr1 mRNA using FISH. We exposed cells to 200 μM H2O2, a concentration that induces apoptosis in approximately half of the cells (31). When cells were exposed to 200 μM H2O2, we observed that approximately half of the cells had strong fluorescent signals (Fig. 3). The signal in the subset of cells not showing egr1 induction at this concentration of H2O2 was indistinguishable from that of control cells. Cells hybridized with sense-oriented probe for egr1 showed no fluorescent signal in any cells (data not shown). Simultaneous double-FISH for egr1 and β-actin mRNA showed no change in β-actin mRNA expression during H2O2 treatment (Fig. 3). These data demonstrate that H2O2 induces a high level of egr1 induction in a subset of cells and no detectable change in egr1 levels in others.

ERK-mediated egr1 Induction and p53 Activation Were Present in Discrete Subsets of Cellular Populations—In many experimental systems, signaling through ERK is known to be prosurvival (16, 30, 49, 50) and p53 activation is known to be proapoptotic (25, 51–53). The p53 protein plays a central role in the cellular response to DNA damage that leads to phosphorylation and activation of p53 (53–55). To characterize the cellular segregation of signaling pathways simultaneously activated by ROS, we monitored the phosphorylation of ERK and p53 signaling pathways after H2O2 exposure using immunocytochemical staining of PC-12-D2R cells. In response to H2O2, enhanced ERK phosphorylation was detected both in the cytoplasm and in the nucleus (Fig. 4A, top). However, phospho-p53 was mainly localized in the nucleus (Fig. 4A, bottom). Approximately half of the cells showed ERK or p53 phosphorylation in response to 200 μM H2O2 (Fig. 4B).

We next used double labeling to study whether there was overlap of p53 and pERK/egr1 induction within the same cells. As shown in Fig. 5A, egr1 mRNA co-localized to cells showing ERK activation. However, the induction of egr1 mRNA by H2O2 was absent in cells showing p53 activation (Fig. 5B). These results indicate that oxidative stress activates ERK or p53 signaling pathways in separate cell subpopulations.

Relative Proportion of Cells Showing Either Activation of ERK or p53 Is Dependent on the Concentration of H2O2 in PC-12-D2R and SN4741 Cells—We have demonstrated previously that PC-12-D2R cells undergo concentration dependent apoptosis when exposed to H2O2 (31). In the present study, we have demonstrated that in undifferentiated PC-12-D2R cells, 200 μM H2O2 activates ERK or p53, and the cells are segregated into separate cell subpopulations. To elucidate the role of these pathways in cell survival or death, we examined the effect of varying concentrations of H2O2 on ERK and p53 activation. As shown in Fig. 6A, low sublethal concentrations of H2O2 activated ERK but not p53. At higher concentrations of H2O2 (200–400 μM), phosphorylation of both ERK and p53 is observed. Consistent with our previous observation that the sublethal concentrations of H2O2 had no significant effect on the cell death (31), the activation of ERK and the absence of p53 phosphorylation by these concentrations of H2O2 suggest that ERK activation is antiapoptotic. At the single cell level, 100 μM H2O2 phosphorylated ERK and induced egr1 in almost all the cells. However, 200 μM H2O2 activated ERK/egr1 in only half of the cells (Fig. 6B).

To explore whether the ROS-mediated segregation of signaling pathways in separate cell subpopulations observed in PC-12-D2R cells was present in a different cellular context, we studied this signaling pathways in a mouse immortalized nigral dopaminergic cell line SN4741 (33). Incubation of these cells with H2O2 was found to induce cell death in a concentration-dependent manner (31, 38). SN4741 cells undergo cell death at low concentrations of H2O2 (50–100 μM) compared with PC-12-D2R cells. We found that H2O2 induced phosphorylation of both ERK and p53 (data not shown). At the single cell level, 50 μM H2O2 phosphorylated ERK and p53 in separate populations of cells (Fig. 7B). However, 100 μM H2O2 activated p53 in almost all the cells (Fig. 7C). These results suggest that ROS activates opposing signaling pathways in discrete cell sub-populations of dopaminergic neuronal cell line.
Total RNA was isolated from PC-12-D2R cells untreated or treated with 200 μM H₂O₂ for 1 h. Changes in the expression level of H₂O₂-induced genes in Affymetrix analysis (n = 4) were independently confirmed using qPCR analysis of RNA from control and H₂O₂-treated cells. The qPCR results are presented as -fold increase over control values using the 2^ΔΔCt formula as described under “Experimental Procedures.” Data presented are mean ± S.E. of one experiment repeated three times with essentially the same results.

**Table I**

| Identifier | Gene description | Microarray Fold change | qPCR Fold change |
|------------|------------------|------------------------|-----------------|
| AF023087   | Nerve growth factor induced factor A (egr1) | 5.66 ± 1.73 | 25.01 ± 1.12 |
| M18416     | egr1             | 4.25 ± 2.18 | 7.83 ± 3.70 |
| U75387     | egr1             | 3.76 ± 6.23 | 5.47 ± 1.12 |
| S81478     | Oxidative stress-inducible protein tyrosine phosphatase (mpk1) | 4.12 ± 5.95 | 1.93 ± 0.30 |
| AA945867   | c-jun            | 4.12 ± 3.20 | 5.50 ± 0.70 |
| AI715959   | c-jun            | 2.58 ± 0.58 | 3.32 ± 3.70 |
| M60921     | NGF-inducible anti-proliferative putative secreted protein (pc3) | 1.63 ± 0.26 | 1.12 ± 0.40 |
| M60921     | pc3              | 1.58 ± 0.40 | 2.34 ± 1.41 |
| AA941456   | pc3              | 2.76 ± 1.99 | 3.20 ± 1.38 |
| AP001417   | Core promoter element binding protein (copeb) | 2.58 ± 0.58 | 3.32 ± 3.70 |
| AI639167   | Expressed sequence tag | 2.33 ± 1.41 | 3.20 ± 1.38 |
| D13123     | F1 mRNA for ATP synthase subunit c | 1.97 ± 0.54 | 3.20 ± 1.38 |
| X06769     | c-fos            | 1.92 ± 0.41 | 2.18 ± 0.50 |
| AA905050   | RhoB             | 1.83 ± 0.19 | 2.18 ± 0.50 |
| L16764     | Heat shock protein 70 (hsps70) | 1.60 ± 0.41 | 3.20 ± 1.38 |

**Fig. 2.** Effect of ERK inhibition on oxidative stress activated gene program. Total RNA was isolated from control PC-12-D2R cells or cells treated with 200 μM H₂O₂ for 1 h in the presence or absence of 100 μM PD98059 (n = 5). The changes in mRNA expression were measured using qPCR analysis. Results are presented as -fold increase over control values using the 2^ΔΔCt formula. Data presented are mean ± S.E. of one experiment repeated three times with essentially the same results. Significant variation between two groups was determined using Student’s t test using Prism software, v. 3.0 (GraphPad Software Inc., San Diego, CA). *p < 0.001 compared with control. #p < 0.001 compared with H₂O₂.

**Activation of ERK and p53 in Postmitotic PC-12-D2R Cells in Response to H₂O₂**—We have previously shown that 200 μM H₂O₂ induce cell death in proliferating and postmitotic PC-12-D2R cells (31). In response to H₂O₂, proliferating cells activate ERK or p53 in separate cell subpopulations. One possible explanation for the opposite responses of individual cells to H₂O₂ could be that cells at different points in the cell cycle might differ in their response. An alternative explanation for our results would be that the initial state differences between cells showing opposite responses are relatively insignificant and the process is stochastic. To determine the influence of cell cycle on the early bifurcation of signaling mechanisms activated by ROS, we differentiated PC-12-D2R cells for 7 days and analyzed the activation of ERK and p53 at the single cell level. As shown in Fig. 8, H₂O₂ (200 μM) activated ERK and p53 in separate populations of cells. These results are similar to those obtained in proliferating PC-12-D2R cells. These results suggest that differences between cells that activate ERK or p53 are independent of cell cycle.

**Caspase-3 Activation Occurs Only in Cells Not Showing ERK Activation in Response to Oxidative Stress**—Double immuno-
used the p53 inhibitor pifithrin-α, which blocks p53 transcriptional activation and subsequent apoptosis (57). In Western immunoblots using phospho-ERK and phospho-p53 antibodies, we have found that pifithrin-α augmented the activation of ERK in presence of H₂O₂ (Fig. 10B). These results suggest that H₂O₂-induced ERK phosphorylation is negatively regulated by activation of p53. However, we found that 40 μM pifithrin-α did not inhibit the phosphorylation of p53 in response to H₂O₂.

To confirm the regulation of ERK by p53, we reduced the levels of p53 expression in PC-12-D₂R cells using RNA interference. After transfection with p53-specific or control siRNA, cultures were assessed for p53 mRNA expression by qPCR. As shown in Fig. 10C, p53 expression was substantially repressed (~4-fold) by 48 h after transfection. The involvement of p53 in oxidative stress-induced ERK activation was studied in PC-12-D₂R cells transfected with p53 or control siRNA. After 48 h of transfection, the cells were incubated with H₂O₂ and assessed for the phosphorylation of ERK. In control siRNA-transfected cells, H₂O₂ activated ERK similar to that observed in cells not transfected with siRNA (Fig. 10D). However, p53 repression by siRNA augmented the ERK phosphorylation (Fig. 10D). These results suggest that cells that activate p53 in response to oxidative stress suppress the activation of ERK.

**Fig. 4. ERK and p53 activation in response to H₂O₂.** A, immunofluorescence microscopy of control PC-12-D₂R cells or cells treated with H₂O₂ (200 μM) for 1 h. Cells were labeled for phosphorylated ERK (p-ERK; red, top) or phosphorylated p53 (p-p53; red, bottom). The nuclei were counterstained with DAPI (blue). B, percentage of PC-12-D₂R cells showing phospho-ERK or phospho-p53 in response to H₂O₂ (200 μM) for 1 h. Cells showing fluorescence phospho-ERK (p-ERK) or phospho-p53 (p-p53) antibodies was scored as phospho-ERK or phospho-p53, respectively, in five experiments. Values represent the mean ± S.E. (200–400 cells scored per experiment).

**Fig. 5. Oxidative stress activates ERK and p53 in different populations of cells.** A, FISH-immunofluorescence microscopy of PC-12-D₂R cells labeled with egr1 mRNA (green) and anti-phospho-ERK (p-ERK; red). B, PC-12-D₂R cells labeled with egr1 mRNA (green) and anti-phospho-p53 (p-p53, red). Cells were incubated with 200 μM H₂O₂ for 1 h. Note that egr1 induction and ERK phosphorylation occur within the same individual cells, whereas egr1 induction and p53 phosphorylation are mutually exclusive. The experiments were repeated four times with similar results.

**Fig. 6. Concentration-dependent H₂O₂ activation of ERK and p53 in undifferentiated PC-12-D₂R cells.** A, cells were treated with the concentrations of H₂O₂ indicated for 1 h. Aliquots of cell extracts were then subjected to immunoblot analysis using antibodies against phospho-ERK (p-ERK) or phospho-p53 (p-p53). The experiments were repeated three times with similar results. B, concentration-dependent activation of ERK at single cell level. Immunofluorescence microscopy of active-ERK (red) in control cells and cells treated with 100 or 200 μM H₂O₂ for 1 h. C, concentration-dependent induction of egr1 at single cell level. FISH for egr1 (green) in control, cells treated with 100 or 200 μM H₂O₂ for 1 h. The nuclei were counterstained with DAPI (blue). The experiments were repeated three times with similar results.
In this study, we demonstrate that within the first hour of cells’ exposure to oxidative injury, they activate specific signaling pathways that indicate whether the cells will ultimately succumb to or tolerate the insult. ERK activation by oxidative stress marks cells that have chosen to maintain homeostasis. In contrast, cells that activate p53 proceed to cell death. Our data indicate that ROS-mediated anti- and pro-apoptotic signaling events are triggered in each cell early after exposure to oxidative stress. These responses are sustained and mutually exclusive. These early, non-overlapping single cell responses are observed in both proliferating and differentiated PC-12-D2R cells and in immortalized dopaminergic SN4741 neurons.

We find that activation of ERK and the induction of egr1 within 1 h mark cells destined to survive after the initial oxidative insult. In PC-12 cells, ERK is mainly activated by growth factors and has been shown to be associated with cell proliferation, differentiation, and promotion of cell survival (13, 16, 58, 59). H2O2 increased the mRNA expression of the ERK-dependent genes egr1, c-jun, c-fos, and mkp1. These genes are known to be transcriptionally activated by increased cellular oxidation (16, 43, 44) and by nerve growth factor (46, 60). Evidence for an antiapoptotic role for ERK has been reported in PC-12 cells after growth factor withdrawal (13, 59) and exposure to oxidative stress (16). ERK has also been reported to function as a suppressor of ROS in superior cervical ganglion neurons (61). Activation of the ERK via the Ras/Raf/MEK pathway has further been shown to support survival of neurons in the nervous system (49, 50).

In contrast to the ERK response, we find that the early activation of p53 by H2O2 predicts the later induction of caspase-3 and apoptosis. The inability of a sublethal dose of H2O2 to activate p53 supports the involvement of p53 in apoptosis in these cells. The p53 tumor suppressor protein has been proposed as a key mediator of stress responses because it plays an essential role in the death of many cell types, including neurons (for review, see Ref. 62). Exposure to ROS can cause nuclear DNA double-stranded breaks that are detected by enzymes from the phosphoinositide 3-kinase family (63), resulting in phosphorylation of serine 15 of p53 and its consequent stabilization and accumulation (40, 41, 54). It has been suggested that modification of this serine regulates p53 stability by altering Mdm2-p53 interactions (40). Activation of p53 results in the up-regulation of proteins implicated in apoptosis (such as proapoptotic BAX and caspase-3) in many experimental systems (25, 51, 52, 64).

We demonstrate that activation of the ERK signaling pathway in response to ROS has no effect on the phosphorylation of p53, whereas p53 inhibition leads to ERK activation. It has been reported previously that ERK activation in response to cisplatin in ovarian cancer cells can phosphorylate p53 in vitro (65). In our experimental system, the pharmacological inhibition of ERK did not affect phosphorylation p53 in response to H2O2. In contrast, we found that inhibition of p53 by pifithrin-α or repression of p53 by siRNA augmented the activation of ERK by H2O2. These results suggest the existence of a negative signaling cross-talk pathway from p53 to ERK. This ERK suppression pathway most likely contributes to the structure of a signaling network switch that forces the cell to rapidly select among these two mutually exclusively patterns of response to oxidative stress.

We find that the recruitment of cells to the response state marked by phospho-p53 increases as the concentration of H2O2 increases and that the early response bifurcation is observed equally in dividing and NGF-differentiated cells. These findings suggest that the initial conditions of the cells that present opposite responses to oxidative stress are likely to be similar.

**DISCUSSION**

**FIG. 7.** Concentration-dependent H2O2 activation of ERK and p53 in SN4741 neuronal cells. Double-immunofluorescence microscopy of anti-phospho-ERK (p-ERK, red) and anti-phospho-p53 (p-p53; green) in untreated cells, cells treated with 50 μM H2O2, or cells treated with 100 μM H2O2, for 1 h. The nuclei were stained with DAPI (blue). Phospho-p53 is concentrated in the nucleus, whereas phospho-ERK is both cytoplasmic and nuclear. Note that phospho-p53 and phospho-ERK are not co-activated within the same individual cells. The experiments were repeated three times with similar results.

**FIG. 8.** Oxidative stress activates ERK and p53 in different sub-populations of neuronally differentiated PC-12-D2R cells. Nerve growth factor-differentiated PC-12-D2R cells were incubated with or without 200 μM H2O2 for 1 h and labeled with anti-phospho-ERK (p-ERK, red) and anti-phospho-p53 (p-p53; green) antibodies. The nuclei were counterstained with DAPI (blue). Top frames, control; bottom frames, H2O2 (200 μM; 1 h). Note that phospho-p53 and phospho-ERK are not co-activated within the same individual cells. The experiments were repeated four times with similar results.
analyzed on agarose gel electrophoresis (A) or by reverse transcription PCR for 30 cycles and qPCR (B). RNA was isolated after 48 h, and p53 mRNA levels were determined by p53-siRNA. The cells were transfected with control or p53-siRNA. Lane 1, control; lane 2, control siRNA; lane 3, p53 siRNA. A, repression of p53 mRNA by p53-siRNA. The cells were transfected with control or p53-siRNA, cells were untreated or treated with H2O2 (200 μM) for 1 h and cell extracts were prepared. Equal amounts of protein were subjected to immunoblot analysis using phospho-ERK or p53 antibodies. The experiments were repeated three times with similar results.

**Fig. 9. p53 activation correlates with caspase-3 mediated apoptosis.** A, double-labeling for phospho-ERK (p-ERK; red) and phospho-p53 (p-p53; green) in PC-12-D2R cells incubated with 200 μM H2O2 for 1 h. Asterisks indicate cells with activated ERK and no phospho-p53. Arrows identify cells with phospho-p53 (green) with no phospho-ERK. B, double-labeling of phospho-ERK (red) and active-caspase 3 (green) in PC-12-D2R cells incubated with 200 μM H2O2 for 24 h. Arrows indicate apoptotic cells with condensed nuclei. Active caspase-3 was localized exclusively in cells with condensed nuclei that are phospho-ERK-negative. The experiments were repeated three times with similar results.

**Fig. 10. Cross-talk between p53 and ERK in response to oxidative stress.** A, PC-12-D2R cells were pretreated with or without 100 μM PD98059 for 1 h and incubated with 200 μM H2O2 for 1 h. B, cells were pretreated with or without 40 μM pifithrin-α for 1 h and incubated with 200 μM H2O2 for 1 h. In A and B, the blots were stripped and reprobed using the antibodies recognizing total ERK or p53 proteins. p-ERK, phospho-ERK; p-p53; phospho-p53. C, repression of p53 mRNA by p53-siRNA. The cells were transfected with control or p53-siRNA, RNA was isolated after 48 h, and p53 mRNA levels were determined by qPCR (graph) or by reverse transcription PCR for 30 cycles and analyzed on agarose gel electrophoresis (bottom). β-Actin from the same samples was amplified as control. Lane 1, control; lane 2, control siRNA; lane 3, p53 siRNA. D, repression of p53 augmented the phosphorylation of ERK in response to H2O2. 48 h after transfection with control or p53-siRNA, cells were untreated or treated with H2O2 (200 μM) for 1 h and cell extracts were prepared. Equal amounts of protein were subjected to immunoblot analysis using phospho-ERK or p53 antibodies. The experiments were repeated three times with similar results.

**Fig. 11. Model of H2O2-induced survival and apoptosis of PC-12-D2R cells.** H2O2-induced ERK functions as antiapoptotic signal to maintain homeostasis of PC-12-D2R cells, whereas p53 activity causes inhibition of ERK and functions as pro-apoptotic signal. The opposite effects of ERK and p53 are brought about by suppression of ERK activation by p53, resulting in an early divergence of the single cell responses toward survival or apoptosis.

For the individual cell, the choice between these two competing and mutually exclusive response states is stochastic. This model, we suggest (Fig. 11), is analogous to the random and exclusive divergence of initially pluripotential cells during development. We propose that the divergent outcome results not from initial differences in the state of the cells but from random selection forced by the design of the oxidative stress signaling circuits. This distinction is important in developing therapeutic strategies to intervene in this process. Our results and interpretation suggest that attempts to identify differences between the subset of cells that survive and those that succumb may be fruitless. On the other hand, further elucidating the structure of the signaling network switch responsible for this bi-stability and determining when the response state becomes unalterable are likely to help in devising rationale strategies to improve the odds for survival of an individual cell.

It has been hypothesized that ROS activates contradictory signaling pathways and that the dynamic balance between these pathways may be important in determining whether a cell survives or undergoes apoptosis (for review, see Ref. 12). Many studies using biochemical assays of cell homogenates have identified the concomitant activation of both pro- and antiapoptotic responses that have served as the basis for models of the underlying mechanisms (e.g. see Refs. 13, 66–69). We find that in PC-12-D2R and SN4741 cells, even at early time points, that divergent responses do not coexist within the same cells. Activation of effector caspases is considered the final step in many apoptosis pathways. Consistent with our findings, it has been reported that caspase activation during apoptosis occurs in an all-or-none fashion (70). Studies using biochemical assays that monitor the average cellular response may obscure the actual decision-making signaling mechanisms by detecting simultaneous responses that are in fact segregated within different cell subpopulations. Our data suggest that “to die or not to die” is a question resolved quickly by each individual cell. Each individual cell rapidly responds to stress and achieves a coherent physiological state directed toward either apoptosis or survival.

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