Alternative, Nonapoptotic Programmed Cell Death

MEDIATION BY ARRESTIN 2, ERK2, AND Nur77*

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Programmed cell death (pcd) may take the form of apoptosis or of nonapoptotic pcd. Whereas cytostatic, caspase-12 and -9, has also recently been described (18–22). Programmed cell death (pcd) is a form of cell death in which the cell plays an active role in its own demise. Although pcd has often been equated with apoptosis, it has become increasingly clear that nonapoptotic forms of pcd also exist (1–13). For example, certain developmental cell deaths, such as "autophagic" cell death (1–5) and "cytoplasmic" cell death (2, 4, 6–9), do not resemble apoptosis. Furthermore, neurodegenerative diseases such as Huntington’s disease and amyotrophic lateral sclerosis demonstrate neuronal cell death that does not fulfill the criteria for apoptosis (10, 11) but instead resembles a recently described form of pcd dubbed paraptosis (12). Ischemia-induced cell death may also display a nonapoptotic morphology, referred to as “oncosis” (13).

The biochemical basis for these alternative morphological forms of cell death remains largely unknown. Understanding the mechanisms for these forms would have potentially important implications for the understanding of evolutionary aspects of cell death programs, developmental cell death, neurodegeneration, and cancer therapies and for the design of novel therapeutic agents for diseases featuring these alternative forms of cell death.

Cell death has been divided into two main types: pcd, in which the cell plays an active role, and passive (necrotic) cell death. The pcd observed during development and tissue homeostasis has been classified morphologically into three main types: type 1, also known as nuclear or apoptotic; type 2 or autophagic; and type 3, also referred to as cytoplasmic (4).

Apoptosis is the best characterized type of pcd, in which the cells display membrane blebbing, flipping of phosphatidylserine in the plasma membrane (14), nuclear fragmentation, and activation of a family of cell suicide cysteine proteases referred to as caspases (15, 16). The biochemical activation of apoptosis occurs through two general pathways: the intrinsic pathway, originating from mitochondrial release of cytochrome c and associated activation of caspase-9, and the extrinsic pathway, originating from the activation of cell surface death receptors such as Fas and resulting in the activation of caspase-8 or -10 (17). A third general pathway, originating from the endoplasmic reticulum and resulting in the activation of caspase-12 and -9, has also recently been described (18–22).

Much less is known about the biochemical mediators of type 2 and type 3 programmed cell death. Type 2 (autophagic) cell death can be activated in some cases by Ras (23), whereas the molecular activation of type 3 cell death is unknown. Recently, it was noted that the binding of the undecapeptide neurotransmitter Substance P (SP) to its receptor, neurokinin-1 receptor (NK1R), induces a nonapoptotic form of pcd resembling type 2 or 3 morphologically and characterized by cytoplasmic vacuolation, lack of caspase activation, lack of inhibition by caspase inhibitors (benzoylcarbonyl-VAD-fluoromethyl ketone and Boc-aspartyl fluoromethyl ketone) and Bcl-xL, lack of nuclear fragmentation or membrane blebbing (Fig. 1), and a requirement for new gene transcription and membrane blebbing (Fig. 1), and a requirement for new gene transcription and translation (24).

The development of specific agonists and antagonists for...
NK₃R has supported a role for NK₃R in numerous biological processes, such as the transmission of pain in the spinal cord. In the central nervous system, it also regulates cardiovascular responses and has recently been implicated in depression and schizophrenia. It appears to be involved in a wide variety of responses due to its ability to modulate the release of other neurotransmitters, such as excitatory amino acids (25).

SP also seems to play an important role in pathological states in which neural cell death occurs, such as status epilepticus and ischemia. For example, SP-null mice demonstrate a resistance to excitotoxin-induced seizures, with an associated reduction in neuronal death (26). Similarly, treatment with an antagonist for NK₃R inhibits seizures and reduces kainic acid-induced cell death in the CA1 region of the hippocampus (27). Furthermore, in a model of focal cerebral ischemia, administration of an NK₃R antagonist reduced infarct volume and improved neurological function (28). Taken together, these observations suggest that NK₃R may be a mediator of cell death in vivo.

In the current report, we demonstrate that SP/NK₃R-induced cell death is mediated by a MAP kinase activation pathway involving Raf-1, MEK2, and extracellular signal-regulated protein kinase 2 (ERK2), upon recruitment by the scaffold protein arrestin 2, in both neuronal and nonneuronal cells. The activation of ERK2 leads to the phosphorylation of Nur77, whose activity is essential for the progression to cell death.

**MATERIALS AND METHODS**

**Neuronal Primary Culture**

Primary striatal cultures were prepared from 17-day-old Sprague-Dawley rat embryos (B&K, Fremont, CA). The tissue was dissected, minced, and trypsinized for 5 min using 0.25% trypsin (Cellgro). After the addition of 10% horse serum to inhibit the trypsin, the cell suspension was triturated 15–20 times with a 10-ml syringe and centrifuged for 5 min at 800 relative centrifugal force. The pellet was resuspended in MEM-PAK (University of California-San Francisco Cell Culture facility), supplemented with 2.02 mg of glucose, 2 mM Glutamax (Invitrogen) and penicillin/streptomycin (100 units/ml). The suspension was filtered through a 70-μm cell strainer, and the final culture medium contained 5% horse serum (Invitrogen). 3–4 × 10⁵ cells/cm² were seeded onto either poly-n-lysine-precocated eight-well chamber slides (BD Biosciences) or 96-well plates precoated with 50 μg/ml poly-n-lysine (Sigma) in water. After a 30-min incubation, unattached cells were removed together with the medium and replaced with glucose-enriched MEM-PAK plus 5% horse serum. The cultures were incubated at 37 °C in 95% air, 5% carbon dioxide with 95% humidity. Cultures were used for experiments between day 1 and day 4 when glial contamination was at a minimum. 0.1 nM to 100 μM SP (Sigma) was added 24–48 h after seeding in the presence of 2.5% horse serum. Viability was quantified by trypan blue staining of the total cell population.

**cAMP Quantification**

Total cellular cAMP was measured using the Biotrak cAMP assay kit (Amersham Biosciences). Single cell imaging was performed in a Merlin imaging facility (Olympus America) using an Olympus IX70 inverted epifluorescence microscope, equipped with a ×40 oil immersion objective and a SpecBright monochromator (Life Science Resources, Cambridge, UK). Excitation, 340 and 380 nm; emission, >505 nm. Cells were loaded with 3 μM Fura-2/AM (from TEF LABS) for 30 min in incubation medium containing 120 mM NaCl, 3.5 mM KCl, 1.3 mM CaCl₂, 0.4 mM KH₂PO₄, 20 mM TES buffer, 5 mM NaHCO₃, 1.2 mM Na₂SO₄, 15 mM glucose, 1.2 mM MgCl₂. pH adjusted to 7.4 with NaOH. 30 μM bovine serum albumin was also added. Experiments were performed in a nonperfusing thermostatted chamber (37 °C) with incubation medium without bovine serum albumin (71). 2 μM ionomycin (Sigma) and 10 μM EGTA (Sigma) were added at the times indicated in Fig. 1B.

**Fig. 1. Morphology of SP/NK₃R-induced Nonapoptotic Programmed Cell Death.** A, 293T cells transfected with NK₃R form vacuole-like structures in response to SP. Semithin sections (1 μm epoxy-embedded) of cells treated with SP (and control, untreated cells) were stained with toluidine blue. The arrows indicate examples of the vacuole-like structures that develop after SP exposure. The image has a × 600 magnification. B, lack of plasma membrane blebbing in 293T cells dying in response to SP/NK₃R. 293T cells were transfected with NK₃R (or exposed or not to SP) or Bax. Note the presence of apoptotic bodies and blebbing in cells expressing the proapoptotic protein Bax, and their absence in SP/NK₃R-induced death. C, lack of nuclear fragmentation in 293T cells dying in response to SP/NK₃R. 293T cells, expressing either NK₃R (with or without SP) or Bax, were stained with 4',6-diamidino-2-phenylindole to compare the nuclear morphologies. The expression of NK₃R was verified by immunostaining (red). Note that in response to SP/NK₃R there is no nuclear fragmentation, whereas such fragmentation is present when the cells express Bax (arrows).

**Inhibitors**

Protein kinase inhibitors were added 30 min before 100 nM SP exposure at the following concentrations: 10 μM adenosine 3',5'-cyclic monophosphorothioate, 8-bromo-, Rp-isomer, sodium salt (Rp-8-Br-cAMPS), which is a protein kinase A inhibitor; 50 μM calphostin C, which is a protein kinase C inhibitor; 10 μM PD 98059 and 10 μM U0126, which are MEK1/2 inhibitors; 10 μM SB203580, which is a p38 inhibitor; and 10 μM SP600125, which is a JNK inhibitor. These compounds were obtained from Calbiochem. The caspase inhibitor Boc-Asp(Ome)CH₂F was obtained from Enzyme Systems Products (Livermore, CA).

**NK₃R Mutants, Plasmids, and Transfections**

NK₃R mutants were created using the QuikChange™ strategy (Stratagene, La Jolla, CA). Using the following sequences for the oligonucleotides: 5'-GGGGCACTGCCTTCGCTTACGCGTCATATTCCATGACGGGTCCAGC-ATTCC-3' for the NK₃R/E78Q mutation and 5'-GCAAACGAAATCCACCCCGATAATTCTTAAATCACGATCACATGC-3' for the NK₃R/R432 truncation. Human embryonic kidney 293T cells were grown in high glucose Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Sigma) and penicillin/streptomycin (100 units/ml) (Invitrogen). The cultures were incubated at 37 °C in 95% air and 5% carbon dioxide with 95% humidity. Transient transfection was performed using Superfect (Qiagen) as described by the manufacturer. Briefly, 2 × 10⁵ cells/well were seeded into 35-mm wells 16 h prior to transfection. Superfect-DNA complexes were added at a 5 μl/2 μg ratio of transfectant; and 10 μl SP was added. Expression of each construct in the transient transfections was determined by Western blot and efficiency of transient transfections by immunocytochemistry (see Fig. 11). Transient transfection efficiencies were in all cases >80%, which was compatible with previously reported...
use of the same system (see Ref. 72; see Figs. 4 and 6 therein). The dominant negative constructs evaluated were shown not to reduce NK1R expression (see Fig. 11). 24–48 h after the addition of SP, cell death was quantified as follows. The media containing floating cells were collected and centrifuged for 5 min at 2000 relative centrifugal force, the pellet was resuspended in PBS/trypan blue (1:10), and the blue cells were counted using a hematocytometer.

Western Blot Analysis

For the transfected HEK293T cells, the cells were washed with cold PBS after incubation with SP for the time indicated and homogenized in lysis buffer (150 μM NaCl, 1% Triton X-100, 50 μM Tris-HCl, pH 8.0, 2 mM Na3VO4, 20 mM NaF, 20 mM glycerol-2-phosphate, proteinase inhibitor mixture (Roche Applied Science)). Cytoplasmic extracts were collected after a 10-min centrifugation at 14,000 relative centrifugal force. Protein was quantified by Bradford assay, and electrophoresis of equal amounts of total protein was performed on SDS-polyacrylamide gels. Separated proteins were transferred to polyvinylidene fluoride membranes at 4 °C for Western blot analysis. Membranes were probed with a 1:1000 dilution of anti-ERK1/2 or anti-phospho-ERK1/2 (BioSource, Camarillo, CA); a 1:8000 dilution of anti-GAPDH (Research Diagnostics, Flanders, NJ); a 1:1000 dilution of anti-MEK1 monoclonal antibody (StressGen Biotechnologies Corp., Victoria, Canada); a 1:1000 dilution of anti-MEK1/2, anti-p38, anti-phospho-p38, anti-JNK1/2, anti-phospho-JNK1/2, or anti-phosphothreonine monoclonal antibody (Cell Signaling Technology, Inc., Beverly, MA); or a 1:200 dilution anti-Nur77 (M-210) (Santa Cruz Biotechnology, Inc., Santa Cruz CA).

Computer programs and data analysis

All data were analyzed using GraphPad Prism (version 6.0, San Diego, CA) and Excel (version 10.0, Redmond, WA). Data represented mean ± S.D. Statistical significance was determined by paired t test analysis or Student’s t test. For Western blot analysis, the error bars represent S.D. (number of independent experiments, n = 3). * p < 0.05 calculated by paired t test analysis.
RNA Interference (RNAi)

Synthesis—The siRNA was generated by in vitro transcription using the Silencer siRNA Construction Kit (Ambion, Austin, TX), following the manufacturer’s instructions. Briefly, for one well (5 × 10⁵ cells/well, 24-well plate format), 1.6 μl of Enhancer R and 100 nm siRNA were diluted in buffer EC to obtain a final volume of 100 μl. After 10 min at room temperature, 4 μl of TransMessenger reagent were mixed gently and incubated for another 10 min at room temperature, and then the complex was diluted with 100 μl of OPTI-MEM. The media were removed from the wells, and the complex mixture was added to the cells and incubated for 3 h at 37 °C, after which time the media were replaced with fresh media and incubated for an additional 20 h. To estimate the efficiency of the transfection, the siRNAs for luciferase (as a sequence irrelevant for cell death) or the NK1R mutants (E78Q) were transfected into cells expressing NK1R. The number of dead cells was quantified by trypan blue exclusion. The error bars represent S.D. (number of duplicated independent experiments, n = 7). ***, p < 0.001; **, p < 0.01 calculated by two-way analysis of variance with Bonferroni post hoc correction.

Microarray Generation and Processing

Custom DNA microarrays were prepared from a Research Genetics human cDNA library (Invitrogen), by amplifying material using PCR from 8432 different cDNAs under standard conditions. This was facilitated through use of a volumetric robot (Genesis, RSF150, Tecan, Durham, NC) and several quality controls to verify the amplification of unique products for each gene, including gel electrophoretic sizing of all amplicons. Amplified products were transferred to 384-well plates and purified using commercially available kits (Millipore Corp., Billerica, MA). The purified PCR products were transferred to 384-well print
plates (Genetix, Beaverton, OR), dried, and resuspended in 6 ml of print buffer (50% Me2SO in 0.04× SSC). High density spotted cDNA microarrays were generated on commercially available glass slides (MWG Epoxy; MWG Biotech Inc., High Point, NC) with a commercial microarayer (Omnigene; GeneMachines, San Carlos, CA) at the Genomics Facility of the Buck Institute. For each printed array, there were a total of 976 buffer controls, which facilitated differentiating signal from noise. The total number of analyzable genes per array, after removing PCR failures and genes with nondetectable expression, was 7483.

Total RNA was prepared from four independent experiments of HEK293T cells transfected with NK1R and treated (or not) with SP for 30 min, 3 h, or 6 h. The RNA quality was assessed by measuring the size distribution on an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA) and by measuring the spectrophotometric 260/280 ratio (>1.8). RNA was labeled with either Cy3 or Cy5 using a commercially available kit (3DNA array 50, Genisphere Inc., Mahwah, NJ).

**Microarray Data Acquisition and Analysis**

After hybridization and washing at high stringency, chips were scanned using a Packard Bioscience scanner (Hewlett Packard, Palo Alto, CA) using ScanArray Express (version 1.1) (PerkinElmer Life Sciences). The scans were carried out using a PMT laser setting of 75 and power of 80. The resulting TIFF files were then analyzed using Quantarray 3 (PerkinElmer Life Sciences) using fixed circle and mean intensity for spot and background quantification. Data were LOWESS-normalized using the GeneTraffic software suite (lobion Informatics, La Jolla, CA). For identifying statistically significant differential expression in the different treatment groups, eight hybridizations per gene (technical duplicates plus experimental duplicates) were analyzed. A list of the genes whose expression presented a mean log2 ratio (experimental/reference) of ≥0.6, with a coefficient of variance (COV = S.D./mean log2 ratio) of <30% were included, giving a total of 91 differentially regulated genes out of 7483.

**RESULTS**

SP/NK1R Death Signaling Is Independent of G-protein Activation—NK1R is a G-protein-coupled receptor that is connected by various second messengers to a wide variety of effector mechanisms to modulate cellular function. Three apparently independent second messenger systems can be activated by G-proteins following ligand binding to NK1R: 1) Ca2+ mobilization from both intra- and extracellular sources via stimulation of phospholipase C; 2) arachidonic acid mobilization via phospholipase A2; and 3) cAMP accumulation via stimulation of adenylate cyclase (25). An alternative pathway, through
interaction with arrestin 2, involves the activation of the MAPK pathway, leading to ERK1/2 activation (29).

We investigated the nature of the signaling pathway activated by SP/NK1R to induce cell death, using the following complementary approaches: 1) RNAi; 2) pharmacological inhibition of specific kinases; and 3) site-directed mutagenesis. Initial experiments, including point mutations in NK1R that reduce the affinity of G-protein interaction (30) or pharmacological inhibition of protein kinase A (Rp-8-Br-cAMPS) or protein kinase C (calphostin C), failed to prevent cell death in response to SP, either in 293T cells overexpressing NK1R (data not shown) or in primary cultures of rat striatal neurons (see below). Similarly, SP induced neither cAMP production in 293T cells overexpressing NK1R (Fig. 2A) nor Ca2+ mobilization in primary rat striatal cells (Fig. 2B).

**SP/NK1R Death Signaling Requires the Involvement of the Arrestin 2 Scaffold Protein—β-Arrestins are scaffold proteins originally described as modulators of receptor endocytosis and mediators of desensitization after prolonged exposure to agonists. Recently, it was discovered that β-arrestins act as signaling adaptors that recruit molecules to the G-protein-coupled receptors in an agonist-dependent fashion. For example, dominant negative forms of arrestin 2 prevented the activation of MAP kinases ERK1/2 by β2-adrenergic receptor; other receptors like protease-activated receptor 2 and angiotensin receptor 1A also interact with arrestin 2 to form multiprotein complexes, and assembly of these complexes leads to the activation of ERK1/2 (31, 32). In the case of NK1R, its carboxyl-terminal region interacts with arrestin 2 following binding of the ligand SP, and a protein complex is formed containing the MAP kinase kinase Raf-1 and the MAPK kinase MEK1/2, leading to the activation of the MAP kinases ERK1/2. SP-stimulated ERK1/2 activation was inhibited by expression of a dominant negative form of arrestin 2 (33). Since SP/NK1R did not induce detectable second messengers originating from G-protein activation (namely cAMP (Fig. 2A) or Ca2+ (Fig. 2B)), and mutations in NK1R that had been shown to affect G-protein signaling had no effect on cell death, we investigated the possibility that the pcd signaling pathway utilized instead the arrestin 2 branch.

As shown in Fig. 3, co-expression of NK1R with a dominant negative form of arrestin 2 in HEK293T cells completely abrogated the cell death in response to SP (for the dominant negative arrestin 2 and for the other co-transfected constructs described below, the expression of NK1R was shown not to be reduced, thus excluding the possibility that the cell death was diminished simply because NK1R expression was decreased nonspecifically by the co-transfections (see “Materials and Methods”).

**Involvement of the MAP Kinase Family Members Raf-1, MEK2, and ERK2 in Nonapoptotic pcd Signaling—**To determine whether arrestin 2 indeed acts as a signaling adaptor coupling the NK1R to MAPK activation, we first determined whether the MAPK kinase kinase Raf-1 participates in this death pathway. Fig. 4 shows the protective role of a dominant negative mutant form of Raf-1 (c-Raf-C4), co-expressed with NK1R in HEK293T cells.

Next, we studied the activation of the MAP kinase proteins MEK1/2 and the MAPK proteins ERK1/2. A mutant NK1R carboxyl-terminally truncated at position 342 (NK1R Δ342) has been reported to exhibit impaired endocytosis (34) and impaired SP-induced desensitization, with enhanced activation of a G-protein pathway increasing intracellular Ca2+ levels (35). Since the MAPK pathway is impaired in the NK1RΔ342 truncation, whereas the ability to activate G-proteins (the other major signaling pathway mediated by NK1R) remains intact, we tested whether NK1RΔ342 is capable of mediating SP-induced cell death. As shown in Fig. 5A, neither NK1RΔ342 nor a NK1R point mutant that has a reduced SP binding (E78Q) (36) induced cell death in response to SP. The level of expression of the mutants was verified by Western blot (data not shown), and the sorting to the plasma membrane was verified by immunocytochemistry (see “Materials and Methods”).

The activation of ERK1/2 by SP in HEK293T cells expressing the wild type NK1R, but not the NK1RΔ342, was verified by Western blot using an antibody recognizing the phosphorylated form of ERK1/2 (Fig. 5B). The activation of ERK1/2 was sustained for at least 6 h, with an apparent second wave of phosphorylation at 3 h of SP exposure (Fig. 5C). In order to determine whether the MAPK pathway activated by SP is indeed required for NK1R-mediated nonapoptotic pcd, we evaluated the effect of the MEK inhibitors PD98059 and U0126. These kinase inhibitors are specific for MEK1/2 and therefore block the activation of ERK1 and ERK2. NK1R-mediated cell death was inhibited by both PD98059 and U0126 (Fig. 5D), which effectively reduced the phosphorylation of ERK1/2 (Fig. 5, B and C). Since the other two MAPK branches, the stress-activated kinases p38 and JNK, had previously been implicated in cell death pathways (37, 38), we investigated whether they were activated in response to SP. As shown in Fig. 5C, neither p38 nor JNK was phosphorylated during the first 6 h of SP death induction.
Consonant with this finding, inhibition of p38 and JNK (using the inhibitors SB203580 and SP600125, respectively) did not inhibit NK,R-mediated cell death (data not shown).

In order to complement the results obtained with pharmacological inhibition of the MAPK pathway and dissect the contributions from ERK1 and ERK2, we utilized an RNAi approach (39, 40). Small interfering RNAs (siRNAs) were designed for ERK1, ERK2, MEK1, and MEK2, targeting regions specific for each gene (i.e., nonconserved among ERK1 and ERK2 or MEK1 and MEK2). Both the ERK2 and MEK2 siRNAs inhibited NK,R-induced cell death, whereas the ERK1 and MEK1 siRNAs had no significant effect on NK,R-induced cell death. GAPDH was included as a control, targeting a gene irrelevant for the process under study (Fig. 6A). Transfection of each siRNA resulted in a decrease in its target of ~80–90% (Fig. 6B), which was similar to the transfection efficiency as judged by the number of transfected cells detected by the use of a fluorescently labeled siRNA (data not shown).

**ERK Activation Also Mediates Nonapoptotic pcd in Striatal Neurons**—Since we had found previously that NK,R-mediated nonapoptotic pcd was observable in neurons in primary culture (24), we evaluated the effects of MEK inhibition on primary neuronal cultures in which cell death had been induced by SP via NK,R (Fig. 7A). The neuronal cell death was completely blocked by PD98059, whereas the addition of the protein kinase C inhibitor calphostin C or the protein kinase A inhibitor Rp-8-Br-cAMPS had no protective effect. These results are compatible with the previous finding of a lack of Ca2+ mobilization in response to SP (Fig. 2B). Preceding cell death in the neurons was an increase in the amount of phosphorylated ERK1/2 after 10–30 min of SP stimulation, whereas the levels of total ERK1/2 remained unchanged (Fig. 7B).

**The Orphan Nuclear Receptor Nur77 Is Up-regulated and Phosphorylated in Response to SP**—In order to identify ERK2 substrates that may mediate nonapoptotic pcd, we searched for genes whose expression was regulated in response to SP by the analysis of microarray hybridizations. From 7,483 transcripts analyzed, 91 were found to be differentially regulated in response to SP. Among the genes induced in response to SP, the orphan nuclear receptor Nur77 (also known as NRB4A1, TR3, NAK-1, and nerve growth factor (NGF)-IB, among other designations) was of particular interest, since it had previously been reported to be phosphorylated by ERK2 but not ERK1 (41) and because it had been shown to be involved in the induction of apoptosis during several different processes, such as T cell negative selection (42), macrophage apoptosis (43), and thapsigargin-induced cell death (44). Nur77 is a member of the thyroid receptor superfamily whose transcription is controlled by external stimuli. It is up-regulated in both T and B cell lymphocytes following antigen receptor ligation, in fibroblasts in the presence of serum, and in neurons in response to NGF stimulation (42). Of potential interest in the context of SP-induced death, it is induced in response to ischemia (45) and kainic acid-triggered seizures (46). Therefore, we hypothesized that Nur77 is an ERK2 target that modulates the SP-induced nonapoptotic cell death program.

To extend the initial observation from microarray studies that Nur77 is up-regulated in response to SP, the accumulation of Nur77 protein following SP stimulation was evaluated by Western blot. As shown in Fig. 8A, Nur77 was barely detectable

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**Fig. 8.** Nur77 is up-regulated and phosphorylated by MAPK in response to SP/NK,R. **A**, time course analysis of Nur77 up-regulation following SP stimulation. Total protein from 293T cells overexpressing NK,R and exposed for the indicated times to SP was analyzed by Western blot using an anti-Nur77 antibody (α-Nur77). The same blot was stripped and reprobed with an antibody against GAPDH to verify similar loading of total protein (α-GAPDH). **B**, Nur77 is phosphorylated on a threonine residue(s). Nur77 was immunoprecipitated from the same samples shown in A, followed by SDS-PAGE and Western blot using an anti-phosphothreonine antibody. C, MEK inhibitors (or RNAi for ERK2) reduced Nur77 threonine phosphorylation but not its induction. Cells overexpressing NK,R were exposed (or not) for a previous 30-min incubation with a previous 30-min incubation of total ERK1/2 remained unchanged (Fig. 7).
in the absence of SP, clearly detectable by 30 min, and was abundant 3 h after the addition of SP.

Nur77 is phosphorylated under different physiological conditions, carried out by different kinases. These include members of the MAPK family activated by NGF/Trk/Ras (47) and Akt (48). Whereas Akt and NGF-induced phosphorylations occur on serine residues (Ser101 for NGF-induced phosphorylation, and Ser350 for Akt), ERK2 phosphorylates Nur77 on threonine 142 (41). Since SP-induced death was dependent on ERK2 activity specifically, we asked whether SP induced Nur77 phosphorylation at a threonine residue or residues. Nur77 was immunoprecipitated from cells exposed for different times to SP. Then the presence of phosphorylated threonine was detected by Western blot, developing with an antibody that recognizes phosphothreonine but not phosphoserine residues. As shown in Fig. 8B, 3 h after SP induction, Nur77 is phosphorylated on threonine(s). This phosphorylation was dependent on ERK activity, since the addition of the MEK inhibitors PD98059 or U0126 prevented such phosphorylation (Fig. 8C).

**Fig. 9. Nur77 activity is essential for SP/NK1R-induced nonapoptotic pcd.** A, co-expression of NK1R with Nur77 dominant negative mutants, either lacking the DNA binding domain (Nur77-ΔDBD) or lacking the trans-activation domain (Nur77-ΔN152), markedly reduced cell death in response to SP, in contrast to the induction of cell death observed when the empty vector was co-transfected (control) with NK1R. The error bars represent S.D. values (number of duplicated independent experiments, n = 3). ***, p < 0.001 calculated by two-way analysis of variance with Bonferroni post hoc correction.

B, decreasing the expression of Nur77 by RNAi reduced the number of dead cells in response to SP/NK1R. 293T cells were transfected with siRNA targeting either GAPDH (siGAPDH) or Nur77 (siNur77), and then NK1R was transfected, and the cells were exposed (or not) for 24 h to SP. The number of trypan blue-positive cells were counted. Error bars, S.D. (number of duplicated independent experiments, n = 3). *, p < 0.05 calculated by paired t test analysis.

C, Western blot to show the efficiency of the siRNA targeting Nur77. 293T cells were transfected with siRNA targeting either GAPDH (siGAPDH) or Nur77 (siNur77) and then transfected with NK1R, and exposed (SP) or not (C) to SP for 3 h. Upper panel, anti-Nur77; bottom panel, the same blot was stripped and reprobed with anti-β-tubulin to confirm equal protein loading.
translocating from the nucleus to the mitochondria, leading to the release of cytochrome c (although how Nur77 mediates this release is unknown). The latter proposed mechanism is based on the lack of inhibition by Nur77ΔDBD, with inhibition instead by a dominant negative mutant that has a deletion of the transactivation domain (Nur77ΔN152) (52).

In the current studies, these dominant negative mutants were co-expressed with NK1R, and the induction of cell death in response to SP was quantified. As shown in Fig. 9A, the overexpression of either the Nur77ΔDBD or the Nur77ΔN152 blocked SP/NK1R-induced death. Dominant negative Nur77 mutants, however, have been shown to inhibit the activities of other Nur77-related molecules, such as Nor1 (55). Therefore, to confirm the specificity of the requirement for Nur77, its expression was silenced by RNAi. As shown in Fig. 9B, interfering with the SP-mediated induction of Nur77 reduced the cell death significantly. The efficiency of the siRNA targeting Nur77 was estimated by Western blot (Fig. 9C).

Taken together, these results suggest that NK1R death signaling is independent of G-protein activation but dependent on an arrestin 2-mediated activation of the MAPK phosphorylation cascade integrated by Raf-1-MEK2-ERK2, leading to the phosphorylation of Nur77 (Fig. 10).

**DISCUSSION**

Three complementary approaches (RNAi, pharmacological inhibition, and site-directed mutagenesis) all implicate arrestin 2, MAP kinase family members, and the orphan nuclear receptor Nur77 as mediators of the nonapoptotic cell death program induced by the NK1R. The finding that arrestin 2 mediates the activation of this nonapoptotic cell death signaling is provocative in light of previous studies of the phototransduction cascade in *Drosophila*, which demonstrated that prolonged accumulation of arrestin 2, complexed with rhodopsin (a G-protein-coupled receptor) in endocytic vesicles, is the initiating factor necessary for photoreceptor cell degeneration (31). Whether arrestin 2 initiates a MAPK cascade in *Drosophila* leading to ERK2-mediated degeneration is still unknown, but it is worth noting that the morphology of the degenerating photoreceptor cells is nonapoptotic and vacuolated, resembling mammalian SP/NK1R-induced death. Recently, Acharya et al. (56) found that modulating a sphingolipid biosynthetic pathway led to rescue of photoreceptor degeneration. Therefore, it would be of interest to determine whether similar sphingolipid modulations affect SP/NK1R-induced death. It is also of interest that several human diseases result from mutations in arrestins. For example, in Oguchi disease, patients develop a degenerative night blindness (57). Thus, the arrestin-modulated molecular pathway of nonapoptotic pcd may be conserved in evolution and may underlie some human diseases featuring cell death.

It is somewhat surprising that MEK2 and ERK2 were implicated in the current studies, since this MAP kinase kinase and MAP kinase have typically been associated with cell survival rather than programmed cell death (58). It is important to note, however, that with the use of specific siRNAs we were able to study individually the role of MEK1 versus MEK2 as well as ERK1 versus ERK2, which had not been possible with the use of pharmacological inhibitors. The fact that only MEK2 and ERK2 mediated cell death suggests that MEK1 and ERK1 may turn out to be the survival/proliferation mediators. If so, then the ratio of ERK1/ERK2 might be a critical determinant of cell fate. The relative toxicity found by the inhibition of MEK1 by RNAi in control cells (Fig. 5) is compatible with the notion that MEK1 may support cellular survival.

In support of our findings, other groups have demonstrated that the activation of ERK1/2 is necessary for neuronal cell death in different paradigms, such as cell death induced by glutamate (59), okadaic acid (60), hemin (61), genistein (62), and 6-hydroxydopamine (63). In addition, another activator of the MAP kinase pathway, Ras, has been implicated previously in caspase-independent pcd (23). Further work is in progress to characterize other downstream targets of ERK2 involved in nonapoptotic pcd and to discern the MEK1/ERK-dependent signals that distinguish a trophic response from a pcd response.

Nur77 as a downstream effector of ERK2 activation (be it direct or indirect) is of potential interest, since its induction has been reported in situations in which neuronal death occurs, such as kainic acid-induced seizures (46) and ischemic brain injury (45). Whether it is acting as a transcription activator or a repressor or is translocated from the nucleus to another organelle (e.g. mitochondria) is under current investigation. Since both dominant negative mutants Nur77ΔDBD (which is located in the cytoplasm and inhibits Nur77 transcriptional activity) and Nur77ΔN152 (which is located in the nucleus and inhibits Nur77-induced cytochrome c release) (52) inhibited SP/NK1R-induced death (Figs. 9 and 11), it was not possible from these results to discern the mechanism by which Nur77 mediates nonapoptotic pcd. The finding that the overexpression of Nur77 dominant negative mutants had a wider inhibition of cell death (close to 100%) than the RNAi of Nur77 (~50%) suggests that other members of the family, such as Nor1 or Nurr1, might also contribute or act redundantly, as has been reported previously (55). A recent report showed that Nur77 induces caspase-independent apoptotic cell death in macrophages (43). In another report, Bcl-2 was found to be unable to inhibit Nur77-mediated T cell death (64). These reports suggest the possibility that Nur77 triggers cell death by...
FIG. 11. The efficiency of the transfection of the NK1R plasmid is not affected by the co-transfection with plasmids encoding proteins that interfere with the death pathway activated by SP/NK1R. HEK293T cells were co-transfected at a ratio of 1:3 with the plasmid encoding NK1R and the following plasmids: GFP (A), dominant negative arrestin 2 (B), Nur77ΔDBD-GFP (C), Nur77ΔN152-GFP (D), or the empty vector (E). The transfected cells were identified by immunocytochemistry using an anti-NK1R antibody (the secondary antibody was Cy3-labeled (red)) or an anti-arrestin 2 antibody (the secondary antibody was coupled to fluorescein isothiocyanate (green)) or by GFP expression. The Nur77 mutants are fused to GFP. The nucleus was stained with 4’,6-diamidino-2-phenylindole (DAPI). The images were magnified × 400.

a mechanism independent of Bax and independent of caspase activation (although it is recognized that these reports were based on different cell death paradigms).

Nur77 also plays a role in cell fate decisions other than as an inducer of cell death; e.g. in response to all-trans-retinoic acid, it may mediate the arrest of cells in the G0/G1 phase of the cell cycle (65). Nur77 has also been reported to act as a death-promoting factor (66) and by tumor necrosis factor (67). Hence, Nur77 may be a pivotal modulator of cell fate, whether related to proliferation or death, either apoptotic or nonapoptotic. The underlying mechanisms that determine which of these outcomes is triggered clearly deserve further investigation.

It has been suggested that SP may also display an anti-apoptotic effect (33, 68). Interestingly, the activation of one death pathway may be associated with the inhibition of another; e.g. some inducers of nonapoptotic pcd such as insulin-like growth factor-I receptor also display an anti-apoptotic effect (12). It has been shown that SP/NK1R may induce mitogenic activity for various cell types including endothelial cells, epithelial cells, human skin fibroblasts, arterial smooth muscle cells, and glioma cells (68, 69), as opposed to cell death induction. Whether signaling via NK1R leads to a mitogenic versus toxic effect may depend on quantitative effects (i.e. hyperstimulation that exceeds some threshold may induce cell death (reminiscent of neuronal excitotoxicity, which also may induce nonapoptotic cell death) (70)) or qualitative effects such as cell state, stimulatory signals, etc. (or both quantitative and qualitative effects). One implication of the current results is that such “trophictoxicity” (i.e. toxicity resulting from activation of a trophic and mitogenic factor receptor) may be involved with cell death, preventing autocrine loop-induced neoplasia. A corollary to this notion is that autocrine loop tumors would be predicted to feature mutations in nonapoptotic pcd-mediating genes.

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Nonapoptotic Programmed Cell Death

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