Activation of Ras Up-regulates Pro-apoptotic BNIP3 in Nitric Oxide-induced Cell Death*

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Nitric oxide (NO) produced by NO synthases causes nitration and nitrosylation of cellular factors. We have shown previously that endogenously produced or exogenously added NO induces expression of BNIP3 (Bcl-2/adenovirus E1B 19kDa-interacting protein 3), leading to death of macrophages (Yook, Y.-H., Kang, K.-H., Maeng, O., Kim, T.-R., Lee, J.-O., Kang, K.-i., Kim, Y.-S., Paik, S.-G., and Lee, H. (2004) Biochem. Biophys. Res. Commun. 321, 298–305). We now report that Ras mediates NO-induced BNIP3 expression via the MEK/ERK/hypoxia-inducible factor (HIF)-1 pathway. (a) Ras-Q61L, a constitutively active form of Ras, up-regulated BNIP3 protein expression by enhancing BNIP3 promoter activity, and ras-S17N, a dominant-negative form, and ras-C118S, an S-nitrosylation mutant, blocked NO-induced BNIP3 expression, suggesting that Ras acts downstream of NO and that NO activates Ras by nitrosylation. (b) U0126, a specific MEK inhibitor, completely abolished BNIP3 expression and the stimulation of promoter activity by NO and Ras, whereas 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, SB203580, and wortmannin, specific inhibitors of soluble guanylyl cyclase, p38 MAPK, and phosphatidylinositol 3-kinase, respectively, had no effect. Ras, MEK1/2, and ERK1/2 were sequentially activated by NO treatment of macrophages. (c) Mutation of the HIF-1-binding site (hypoxia-response element) in the BNIP3 promoter abolished BNIP3 induction, and HIF-1α was strongly induced by NO. (d) Transient expression of activated Ras promoted macrophage death, as did NO, and this Ras-mediated cell death was inhibited by silencing BNIP3 expression. These results suggest that NO-induced death of macrophages is mediated, at least in part, by BNIP3 induction.

Macrophages play an indispensable role in protecting organisms from invading pathogens. When activated by pathogenic microorganisms, they produce various immune mediators, including a burst of nitric oxide (NO) via inducible NO synthase (1). The released NO is cytotoxic and essential for destroying bacteria. It is also toxic to host cells, including the macrophages themselves (2, 3), and macrophages have been used as models of NO-induced apoptosis. NO-induced apoptosis is accompanied by cytochrome c release, loss of mitochondrial transmembrane potential, and extensive cleavage of poly(ADP-ribose) polymerase (4–6). There is also evidence for caspase-independent NO-induced cell death pathways (7).

Ras was first identified as the product of an oncogene promoting cell cycle progression (8). Mutations in the ras gene have been identified in numerous human cancers (9), but ras, like other oncogenes, can also induce apoptosis or cell cycle arrest (10, 11). Activated Ras binds to RASSF, Nore1, and MST1, inducing apoptotic or tumor-suppressing signals. The Ras/Raf/MEK2/ERK signaling pathway can induce either apoptosis or cell cycle progression, whereas the Ras/PI3K/Akt pathway usually evokes cell survival signals and prevents apoptosis. Ras activation and inactivation are catalyzed by guanine nucleotide exchange factors and GTPase-activating protein. Ras can also be activated by S-nitrosylation at Cys118, which promotes GDP/GTP exchange (12–15). The NO modification of Ras has the same effect as the binding of guanine nucleotide exchange factors to Ras and can lead to stimulation of down-stream signaling pathways. Ras is a physiological target of endogenously produced NO through NO synthase (16), but the physiological role of NO-activated Ras remains to be identified.

BNIP3 (Bcl-2/adenovirus E1B 19kDa-interacting protein 3) was first discovered in a yeast two-hybrid screen as interacting with the adenovirus E1B 19-kDa protein, a homolog of Bcl-2 (17). BNIP3 is a pro-apoptotic transmembrane protein that is inserted in the outer mitochondrial membrane and belongs to the BH3 (Bcl-2 homology 3) domain-only subfamily (18, 19). A mutant form lacking its transmembrane domain and C-terminal region is not localized to mitochondria and loses its pro-apoptotic activity (18, 20, 21). Therefore, the transmembrane domain of BNIP3, but not the BH3 domain, is required for mitochondrial targeting and pro-apoptotic function. Overexpression of BNIP3 leads to opening of the mitochondrial per-
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meability transition pore, thereby abolishing the proton electrochemical gradient, and this is followed by chromatin condensation and DNA fragmentation (22, 23). BNIP3 can be induced by hypoxia and plays a role in hypoxic cell death in many cell lines derived from carcinomas, macrophages, and endothelial cells (24, 25). Expression of BNIP3 in hypoxic cells is regulated mainly at the level of transcription and is under the control of the transcription factor HIF-1. In addition, BNIP3 is overexpressed even in some well vascularized tumors and non-hypoxic cells (25), suggesting that there may be BNIP3-inducing stimuli other than hypoxia. Several candidate factors that can increase BNIP3 expression have been reported recently. Pleomorphic adenomas gene-like 2 (PLAGL2) protein induces death of Balb/c 3T3 fibroblasts and neuroblastoma cells and promotes the accumulation of BNIP3 (26). BNIP3 has also been shown to be involved in CD47-induced apoptosis of Jurkat T cells and in activation-induced death of effector cytotoxic T lymphocytes (27, 28).

We showed recently by microarray analysis that endogenously produced or exogenously added NO activates the Bnip3 promoter and induces expression of BNIP3 protein in RAW264.7 macrophages under normoxic conditions (29). Peritoneal macrophages from NO synthase-null mice fail to produce BNIP3 in response to lipopolysaccharide, and overexpression of BNIP3 leads to apoptosis of the macrophages. These data led to the conclusion that BNIP3 is involved in NO-induced macrophage death. In this study, we report that BNIP3 induction by NO is mediated by Ras, followed by MEK, ERK, and HIF-1. Ras activation also induces death of macrophages, and antisense silencing of BNIP3 expression greatly reduces death, suggesting that it results from up-regulation of BNIP3.

EXPERIMENTAL PROCEDURES

Cell Culture—Mouse RAW264.7 macrophages and human embryonic kidney HEK293T cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (HyClone, Logan, UT) and antibiotics/antimycotics (Invitrogen). Cells were grown at 37 °C in a humidified atmosphere of 5% CO₂.

Construction of Expression Plasmids—Human and mouse Bnip3 cDNAs were prepared from total RNAs of HEK293T cells and RAW264.7 macrophages, respectively. The total RNAs were reverse-transcribed, and Bnip3 was amplified by PCR and cloned into pcDNA3.1(+) (Invitrogen) as described previously (29). The nucleotide sequences of the primers were based on BCO46603 (mouse; GenBank™ Data Bank) and AL162274 (human; GenBank™ Data Bank). The pcDNA3.1(+) vector was used to clone an antisense Bnip3 cDNA. The putative mouse Bnip3 promoter region was searched with NT_039435 (mouse chromosome 7; GenBank™ Data Bank). A promoter fragment (bp −636 to −1; +1 indicates the translation start site) was amplified with primers 5’-ACTACAGATTCGTCGAGGAGGCAGACGAGAACC-3’ (forward) and 5’-ATCCACATGTTGCTCGGCGCAAAAGGAGCC-3’ (reverse) and mouse genomic DNA as a template, and the product was cloned upstream of luc²⁺ in the pGL3-Basic vector (Promega Corp., Madison, WI). 5’-Serial deletion constructs were generated by PCR using appropriate primers and the 636-bp Bnip3 promoter as template, followed by subcloning into the pGL3-Basic vector. Analysis of the putative Bnip3 promoter sequences for transcription factor-binding sites was done with the MatInspector program (Genomatix Software GmbH). To mutate the HIF-1-binding site (hypoxia-response element), Egr-1-binding site, and p53-binding site in the region from bp −249 to −214 of the Bnip3 promoter, point mutations were introduced into the pGL3/281-bp Bnip3 promoter by overlap PCR. Mutations of the binding sites were prepared (underlined and in boldface): wild-type HIF-1, GGGCGCGACACGTGCC; mutant HIF-1, GCAGCGCGACACGT GCC; wild-type Egr-1, GCCGGCGCGAGACG GCC; and mutant Egr-1, GCCGGCGCGACACGT GCC; and mutant p53, GCCAAATCCGGCAGACTCCGCC. Vectors expressing pTRE-ras-Q61L, pTRE-ras-S17N, and pcDNA3.1(+)−ras-C118S were kind gifts of Dr. Hye-Young Yun (Chung-Ang University, Seoul, Korea) (30). The pcDNA3.1(+)−ras-Q61L and pcDNA3.1(+)−ras-S17N vectors were constructed by cloning the corresponding insert into the BamHI site of the pcDNA3.1(+) vector. To clone Pro-GFP-BNIP3, the PCR-amplified product obtained using GFP-BNIP3 and primers 5’-ACCGCTCGCCACACTGTTGAG-3’ (forward) and 5’-GTGATTCTAGACGGTGAGTTGC-3’ (reverse) was subcloned into the vector for the pGL3/281-bp Bnip3 promoter after digestion with NcoI/XbaI. In this way, the luciferase reporter gene was replaced with GFP-BNIP3, as depicted in Fig. 5A. All constructs were confirmed by sequencing.

RT-PCR Analysis—Total RNA was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (Promega Corp.), and semiquantitative PCR was performed with the following primer pairs: mouse BNIP3, 5’-AGAACCTGCAAGGATGG-3’ (forward) and 5’-GAAGTTTCATGTCGAAGGCCC-3’ (reverse); human BNIP3, 5’-CCCGGGATGTGGGGCGCC-3’ (forward) and 5’-GTGGTTCACCGACTGGAGCCTGCACC-3’ (reverse). PCR products were resolved by electrophoresis on 1% agarose gels and ethidium bromide staining. All reactions were performed in duplicate.

Assay of Cell Viability—Cells were plated in triplicate at 1 × 10⁴ cells/well in 96-well plates, treated as desired, and incubated for 12, 24, 36, and 48 h at 37 °C. For the last 4 h of incubation, 50 µl of 5 mg/ml MTT (Sigma) was added to each well. The plates were centrifuged; the supernatants were carefully aspirated; and 100 µl of 0.04 N HCl/isopropyl alcohol was incubated in each well for 10 min. Absorbances were recorded with a microplate reader (Molecular Devices) at 540 nm. For propidium iodide staining of apoptosis, cells were gently harvested using 0.05% trypsin, washed with phosphate-buffered saline, and stained with 100 µg/ml propidium iodide. The percentage of cell death was measured by counting propidium iodide-stained cells with a FACScan (BD Biosciences). Data were analyzed with ModFitLT Version 3.0 software.

Western Blot Analyses—Western blot analyses were performed as described previously (29). Briefly, cultured cells (5 × 10⁶ cells/100-mm culture dish) were washed with phosphate-buffered saline and lysed in lysis buffer (50 mM Tris-
HCl, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride (pH 7.5), and freshly added protease inhibitor mixture (Sigma)). The cells were incubated on ice for 20 min and centrifuged at 15,000 rpm for 10 min at 4 °C. To prepare nuclear extracts, the cells were incubated on ice for 15 min in 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride and centrifuged at 15,000 rpm for 5 min at 4 °C. The pellet was incubated in lysis buffer on ice for 30 min. After centrifugation, the protein concentration of the supernatant was determined by BCA assay (Pierce). Samples containing 20 μg of protein were resolved on 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) in a Mighty Small Transphor unit (Amersham Biosciences). For Western blotting of BNIP3 protein, equivalent numbers of cells were lysed in SDS-PAGE loading buffer containing 0.2M MgCl2. Equal loading was verified by SDS-PAGE and staining with Coomassie Brilliant Blue. The primary antibodies used were as follows: anti-human HIF-1α (catalog no. NB 100-123) from Novus Biologicals (Littleton, CO); anti-Ras (clone RAS10) from Upstate (Lake Placid, NY); anti-ERK1/2 (catalog no. 9102), anti-phospho-ERK1/2 (catalog no. 9101), anti-MEK1/2 (catalog no. 9122), and anti-phospho-MEK1/2 (catalog no. 9121).
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from Cell Signaling Technology, Inc. (Danvers, MA); anti-BNIP3 (catalog number ab10433) from Abcam (Cambridge, UK); and rabbit anti-human p53 (catalog no. sc-6243), anti-cyclin D1 (catalog no. sc-7353), anti-Bad (catalog no. sc-7869), and anti-Bax (catalog no. sc-493) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-tubulin monoclonal antibody (catalog number T5168) was from Sigma.

Ras Activity Assay—Ras activity was measured with a Ras activation assay kit (Upstate) detecting Ras bound to the Ras-binding domain of Raf-1, which specifically binds GTP-bound Ras. Briefly, RAW264.7 cells were washed with cold phosphate-buffered saline and lysed for 30 min in the kit lysis buffer with freshly added protease inhibitor mixture. Active Ras was precipitated using a glutathione S-transferase fusion of the Raf-1 Ras-binding domain bound to glutathione-agarose beads by incubation for 45 min at 4 °C with gentle agitation. Western blotting with anti-Ras antibody (clone RAS10) was used to detect bead-bound activated Ras. Positive and negative controls were prepared by in vitro labeling of samples with either GTPγS or GDP, respectively, prior to affinity precipitation (data not shown).

Transient Transfections—Transfection was performed with Transfectin™ lipid reagent (Bio-Rad). For reporter assays, 1.5 × 10⁵ cells were seeded in 12-well plates at least 12 h before transfection. Cells were transfected with 2 μg of reporter plasmids and 0.3 μg of pRL-TK (an internal control plasmid expressing the Renilla luciferase gene; Promega Corp.). For cotransfection in reporter assays, 0.5 μg of reporter plasmids

FIGURE 3. Activation of MEK, ERK, and Ras proteins by NO. A and B, RAW264.7 cells were cultured with 100 μM SNAP for the indicated times (0–3 h). 20 μg of total cell protein (A) or nuclear extract protein (B) was subjected to Western blot analysis and probed with antibodies against MEK and phospho-MEK (pMEK) and with monoclonal antibodies against ERK and phospho-ERK (pERK), respectively. C, cells were pretreated with 10 μM U0126 (U), 2 μM SB203580 (SB), or 2 μM wortmannin (Wort) for 15 min and then incubated with 100 μM SNAP for 1 h for detection of phospho-ERK or for 12 h for detection of BNIP3. Nuclear extracts or total cell extracts were analyzed by Western blotting using anti-phospho-ERK or anti-BNIP3 antibody, respectively. Expression of α-tubulin is shown as an internal standard for BNIP3 expression. Similar results were obtained in three independent experiments. Ctrl, control; D, cells were treated with 100 μM SNAP for the indicated times (0–3 h), 30 μg of total cell extract was incubated with the glutathione S-transferase-fused Raf-1 Ras-binding domain on glutathione-agarose. The captured protein was eluted and analyzed by Western blot (WB) analysis using anti-Ras monoclonal antibody. In a parallel experiment, 20 μg of total cell lysate was directly subjected to Western blot analysis and probed with anti-Ras antibody for detection of total Ras protein. IP, immunoprecipitation.

FIGURE 4. Ras induces Bnip3 promoter activity via the MEK pathway. A, HEK293T cells on 100-mm plates were transiently transfected with 20 μg of expression vectors for the ras mutants as indicated for 24 h. Total cell lysates were analyzed by Western blotting with anti-Ras antibody. NSB indicates nonspecific bands as internal controls. Mock vector (pcDNA3.1(+))-transfected cells were used as a negative control. B, RAW264.7 macrophage cells in 12-well plates were transiently transfected with 0.5 μg of 281-bp Bnip3 promoter-luciferase vector, 0.3 μg of pRL-TK (Renilla luciferase) vector, and various concentrations of expression vectors for the ras mutants for 24 h. The total concentrations of DNA in each sample were adjusted to be the same using the mock vector. C, fold induction of firefly luciferase is shown relative to the cells transfected with mock vectors along with the reporter plasmids for the Bnip3 promoter. Error bars indicate the means ± S.E. of three independent transfection experiments. D, the cells were transiently transfected with 0.5 μg of 281-bp Bnip3 promoter-luciferase vector, 0.3 μg of pRL-TK vector, and 1.5 μg of ras-Q61L vector and incubated in the absence or presence of 10 μM U0126 (U), 2 μM SB203580 (SB), or 2 μM wortmannin (Wort) for 24 h. The total concentrations of DNA in each sample were adjusted to be the same using the mock vector. D, the cells were transiently transfected with 0.5 μg of 281-bp Bnip3 promoter-luciferase vector, 0.3 μg of pRL-TK vector, and 1.5 μg of the indicated vectors for the ras mutants for 24 h. For the last 12 h of transfection, the cells were incubated with 0, 50, 100, 200, and 500 μM SNAP. Fold induction of firefly luciferase is shown relative to cells transfected with mock vectors along with reporter plasmids without SNAP treatment. Error bars indicate the means ± S.E. of three independent experiments.
serum, 0.1% saponin, and 0.1% NaN₃ with phosphate-buffered saline for 15 min at room temperature. After staining with 10 μg/ml Hoechst 33258 (Sigma) for 5 min, the cells were mounted on slide glasses. Fluorescent images were acquired with a Zeiss confocal microscope.

RESULTS

NO Induces BNIP3 Expression—Induction versus suppression of apoptosis by NO is largely concentration- and cell type-dependent. We determined previously the concentration of SNAP (an NO donor) equivalent to the concentration of endogenous NO released by activated RAW264.7 macrophages (29). We used lipopolysaccharide/interferon-γ as an endogenous NO inducer because lipopolysaccharide/interferon-γ increased the expression of inducible NO synthase and caused the release of endogenous NO; ∼20 μM nitrite accumulated in the culture medium after exposure to 500 ng/ml lipopolysaccharide for 24 h, and a similar amount of nitrite was generated by 100 μM SNAP under the same conditions. In this study, we used 100 μM SNAP to treat the RAW264.7 cells unless indicated otherwise. Cells were exposed to SNAP for 3–24 h, harvested, and subjected to RT-PCR (Fig. 1A). Induction of Bnip3 mRNA reached a maximum after 9 h of SNAP treatment. BNIP3 protein increased in a dose-dependent manner between 100 and 500 μM and reached a maximum at 12 h (Fig. 1B). We have shown previously that NO stimulates Bnip3 promoter activity and that the NO-responsive sites of the mouse Bnip3 promoter are located within the 281-bp region upstream of the translation initiation site. The activity of this 281-bp Bnip3 promoter region was also induced in a dose-dependent manner by SNAP (Fig. 1C). Evidently NO-induced BNIP3 protein expression is accompanied by increased transcript levels and promoter activity, suggesting that NO regulates BNIP3 expression at the level of transcription.

BNIP3 Induction Is Not Controlled by Guanylyl Cyclase but by the MEK Signaling Pathway—We examined whether NO induces BNIP3 expression via cGMP because NO activates soluble guanylyl cyclase (31). When the cells were pretreated with various concentrations of 1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one, a specific soluble guanylyl cyclase inhibitor, NO-in-
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A. Relative luciferase activity

B. The region from bp −226 to −213 of the Bnip3 promoter is responsible for NO-induced activity. A, deletions of the mouse Bnip3 promoter were made as described under “Experimental Procedures.” RAW264.7 macrophages were transfected with 2 μg of each of the 5′-deletion constructs and 0.3 μg of pRL-TK (Renilla luciferase (Luc)) vector for 24 h and cultured in the absence or presence of 100 μM SNAP during the last 12 h. Luciferase activity was normalized by Renilla luciferase expression and is shown as fold induction relative to the promoter activity of cells transfected with the indicated promoter without SNAP treatment. Data are the means ± S.E. of three samples. Similar results were obtained in three independent experiments. B, shown is a schematic representation of the 281-bp Bnip3 promoter. Consensus HIF-1-, p53-, and Egr-1-binding sites exist in the region bp −249 to −214. The translation start codon of the Bnip3 protein is marked as +1.

FIGURE 6. The region from bp −226 to −213 of the Bnip3 promoter is responsible for NO-induced activity. A, deletions of the mouse Bnip3 promoter were made as described under “Experimental Procedures.” RAW264.7 macrophages were transfected with 2 μg of each of the 5′-deletion constructs and 0.3 μg of pRL-TK (Renilla luciferase (Luc)) vector for 24 h and cultured in the absence or presence of 100 μM SNAP during the last 12 h. Luciferase activity was normalized by Renilla luciferase expression and is shown as fold induction relative to the promoter activity of cells transfected with the indicated promoter without SNAP treatment. Data are the means ± S.E. of three samples. Similar results were obtained in three independent experiments. B, shown is a schematic representation of the 281-bp Bnip3 promoter. Consensus HIF-1-, p53-, and Egr-1-binding sites exist in the region bp −249 to −214. The translation start codon of the Bnip3 protein is marked as +1.

duced Bnip3 expression was not affected (Fig. 2A), suggesting that it is not controlled by the soluble guanylyl cyclase/cGMP pathway. Next, we tested the roles of the MAPK and PI3K pathways, which are among the major signal transduction pathways induced by growth factors, cytokines, or stress. Cells were pretreated with U0126, SB203580, or wortmannin (or LY294002), inhibitors of MEK, p38 MAPK, and PI3K, respectively. Only U0126 inhibited Bnip3 induction (Fig. 2B). Pretreatment with U0126 also blocked luciferase expression from the 281-bp Bnip3 promoter, whereas SB203580 and wortmannin had barely any inhibitory effect (Fig. 2C).

To investigate whether the MEK pathway is activated by SNAP, we performed Western blotting with anti-phospho-MEK1/2 and anti-phospho-ERK1/2 antibodies. Phosphorylation of both MEK1/2 and ERK1/2 was detected within 30 min (Fig. 3, A and B), with a peak between 1 and 2 h. The effect of U0126 treatment on NO-induced Bnip3 protein expression was also examined. U0126 treatment completely inhibited phosphorylation of ERK1/2 and reduced expression of Bnip3 protein to its basal level (Fig. 3C). Because Ras is an upstream regulator of the MEK/ERK pathways (8, 9) and can be activated by S-nitrosylation (13–16), we analyzed Ras activity in the NO-treated cells. Pulldown assays using the Ras-binding domain of Raf-1 revealed a marked increase in activated GTP-bound Ras within 30 min of SNAP treatment, whereas total Ras expression was not changed (Fig. 3D). These results indicate that NO-induced Bnip3 expression is controlled by the MEK/ERK pathway and suggest that Ras is an upstream regulator.

Ras Induces Bnip3 Expression via the MEK Pathway—The involvement of Ras in NO-induced Bnip3 expression was confirmed using plasmids bearing constitutively active ras (Q61L mutant), dominant-negative ras (S17N mutant), and mutant ras with the major S-nitrosylation site changed to serine (C118S mutant). The expression levels of the mutant Ras proteins in the transfected HEK293T cells were comparable (Fig. 4A). When we measured the promoter activity of Bnip3 in cells transfected with the ras mutant vectors and the Bnip3-luciferase reporter plasmid, the ras-Q61L mutant activated the Bnip3 promoter, whereas the ras-S17N and ras-C118S mutants had no effect (Fig. 4B).

Next, we tested whether Ras activation is on the same pathway as NO activation. As expected, U0126, which inhibits NO-induced Bnip3 expression, also blocked Ras-induced Bnip3-luciferase reporter activity, whereas SB203580 and wortmannin have hardly any inhibitory effect (Fig. 4C). We also found that mutants ras-S17N and ras-C118S inhibited Bnip3 induction by NO when they were transfected into the cells followed by treatment with SNAP. Although ras-C118S is not a dominant-negative mutant, its ectopic expression at high levels seems to prevent endogenous Ras from signaling, as shown previously (13, 32). As expected, ras-Q61L was not inhibitory but rather had an additive effect on NO-dependent induction of Bnip3 promoter activity (Fig. 4D). In the ras-Q61L-transfected cells, high concentrations of SNAP did not have a clear-cut concentration-dependent effect on the induction of Bnip3 promoter activity, probably because some of the ras-Q61L-transfected cells died at those high doses of SNAP, as discussed below. Collectively, these results suggest that activation of Bnip3 promoter activity by Ras probably occurs by the same signaling pathway as that by NO. Ras seems to act downstream of NO to induce Bnip3 expression, and NO may act by nitrosylating Ras.

Next we examined whether Bnip3 protein is induced by Ras activation. We have shown previously that a GFP-Bnip3 fusion protein has the same effect as Bnip3 in inducing apoptosis of macrophages (29). We constructed a plasmid (Pro-GFP-Bnip3) in which GFP-Bnip3 is under the control of the 281-bp Bnip3 promoter, as depicted in Fig. 5A. RAW264.7 cells were transfected with Pro-GFP-Bnip3 and ras-Q61L with or without SNAP treatment, and GFP-positive cells were counted.
GFP-BNIP3 was completely absent from nuclei (Fig. 5A), in accord with the previous results. In mock-transfected cells not exposed to SNAP, the number of GFP-positive cells was very low. As expected, this number increased (~8-fold) in response to 100 μM SNAP (Fig. 5B), and cotransfection with ras-Q61L also enhanced GFP-BNIP3 expression. When the cells were exposed to SNAP and transfected with ras-Q61L, GFP-positive cell numbers increased even more, demonstrating an additive effect of the two upstream signaling effectors.

Next, we studied the induction of endogenous BNIP3 protein by transfection of RAW264.7 cells with the ras-Q61L expression plasmid. Expression of ras-Q61L strongly induced BNIP3 20–30 h after transfection (Fig. 5C). Because the anti-proliferative effect of Ras is often associated with induction of p53 and down-regulation of cyclin D1 (33–35), we characterized the expression of these target molecules as well as of the pro-apoptotic Bcl-2 family proteins Bax and Bad. Western blot analysis showed that expression of ras-Q61L did not alter the levels of p53, cyclin D1, Bax, and Bad, demonstrating that the induction of BNIP3 expression by Ras activation is specific. We also introduced the ras-Q61L plasmid by transfection into HEK293T cells. As shown in Fig. 5D, activated Ras strongly induced BNIP3 mRNA and protein in these cells. Together, these results show that Ras activation strongly induces Bnip3 promoter activity and expression of Bnip3 mRNA and protein.

**HIF-1 Is Responsible for NO- and Ras-dependent Induction of BNIP3 Transcription**—To identify the cis-acting elements in the 281-bp Bnip3 promoter, we made serial deletions of the promoter, as depicted in Fig. 6A. Whereas deletion of the region from bp −636 to −226 did not affect promoter activity, deletion of the region from bp −226 to −213 completely abolished the NO responsiveness of the promoter (Fig. 6A). Because this region harbors the consensus sequences for binding of HIF-1 and p53 and is close to the consensus sequence for binding of Egr-1 (Fig. 6B), we made point mutations in these sites (see “Experimental Procedures”). Mutation of the HIF-1-binding site (hypoxia-response element) dramatically reduced Bnip3 promoter activity in response to SNAP, whereas mutation of the Egr-1 and p53 sites had no effect (Fig. 7A). Bnip3 promoter activation by Ras also depended on the HIF-1-binding sites (Fig. 7B). Based on these results, we propose that HIF-1 is the transcription factor responsible for NO- and Ras-dependent induction of Bnip3 transcription. We also tested whether SNAP treatment increases HIF-1α expression. HIF-1α protein began to increase within 3 h of SNAP treatment and reached a peak after 9 h (Fig. 8A), and this effect was abolished by pretreatment with U0126 (Fig. 8B). Taken together, these data indicate that HIF-1α is the downstream transcription factor in the MEK signaling pathway leading to Bnip3 expression and that a pathway linking NO, Ras, MEK, and HIF-1 is responsible for inducing the expression of pro-apoptotic Bnip3.

**Effects of Ras Activation on the Growth of RAW264.7 Macrophages**—We next investigated whether expression of Bnip3 induced by Ras activation is reflected in the death of RAW264.7 cells. When the cells were harvested, we consistently found that the recovery of total protein from RAW264.7 cells following ras-Q61L transfection was lower than that following mock vector transfection. Therefore, we transfected cells with mock or ras-Q61L expression vectors and measured cell viability at various times thereafter. There was a significant loss of viability of the ras-Q61L-transfected cells (Fig. 9A). For example, after 48 h of transfection, we observed a 40% reduction of cell viability in ras-Q61L-transfected cells compared with mock-transfected cells. Considering the moderate efficiency of transfection of the RAW264.7 cells, this loss of viability is quite striking. Transfection with ras-S17N or ras-C118S had no effect, showing that the reduction of viability due to activated Ras is specific. Consistent with previous observations, the SNAP-treated cells retained only 40% viability after 48 h of incubation. The number of propidium iodide-positive cells also increased significantly within 24 h in ras-Q61L-transfected or SNAP-treated cells (Fig. 9B). Therefore, most of the reduction in the number of viable cells was not due to reduced proliferation but to induction of cell death. These results suggest that Ras activation antagonizes the survival of RAW264.7 cells.

To confirm that BNIP3 acts as a downstream regulator of Ras-induced cell death, we tested whether silencing of BNIP3 expression affects cell death. An antisense Bnip3 DNA construct (see “Experimental Procedures”) was cotransfected with ras-Q61L, and the expression of BNIP3 protein was examined 20–30 h after transfection. As shown in Fig. 10A, BNIP3
expression was abolished. Similar results were obtained with ras-Q61L/antisense Bnip3 plasmid ratios of 1:1, 1:3, and 1:5.

We then examined whether silencing of Bnip3 expression influences Ras-induced cell death. Cells were transfected with a 1:1 mixture of ras-Q61L and the antisense Bnip3 construct and compared with cells transfected with ras-Q61L or mock vector only. Bnip3 silencing almost completely rescued the cells from Ras-induced death (Fig. 10B). For example, after 48 h, the viability of the ras-Q61L-transfected cells was 65%, whereas that of the ras-Q61L/antisense Bnip3-transfected cells increased to 92%. Similar results were obtained with an optical microscope: an increased number of attached live cells were obvious after cotransfection with ras-Q61L and the antisense Bnip3 vector.

Finally, we tested whether silencing of Bnip3 expression and inhibition of Ras activation also affect NO-induced cell death. As shown by the cell viability assays in Fig. 10C, transfection of the antisense Bnip3 or dominant-negative ras-S17N construct rescued 20–45 and 15–25%, respectively, of the cells from NO-induced death 36 h after transfection. In view of the moderate efficiency of transfection of the RAW264.7 cells, this increase in viability is quite significant. Propidium iodide staining of apoptotic cells gave similar results (data not shown). These findings indicate that Bnip3 induction via the activated Ras pathway is responsible, at least in part, for the death of macrophages induced by NO.

**DISCUSSION**

In this study, we analyzed the signaling pathways that regulate Bnip3 expression to identify the cell death pathway induced by NO in RAW264.7 cells. We first found that NO-induced Bnip3 expression is controlled via a MEK/ERK/HIF-1 signaling pathway based on the following evidence. (a) Treatment with U0126, a MEK inhibitor, abolished expression of Bnip3 mRNA and protein, whereas inhibitors of soluble guanylyl cyclase, p38 MAPK, and PI3K had no effect (Figs. 2 and 3). (b) Bnip3 promoter activity, which can be induced by NO treatment, was also reduced to basal levels by co-treatment with U0126 (Fig. 2); (c) the HIF-1-binding site at bp -226 to -214 is responsible for NO-dependent induction of Bnip3 promoter activity (Figs. 6 and 7). (d) MEK1/2, ERK1/2, and HIF-1α were strongly activated by NO treatment, and the expression of phospho-ERK1/2 and HIF-1α was also reduced to basal levels by co-treatment with U0126 (Figs. 3 and 8). This conclusion is consistent with previous evidence that NO activates HIF-1 in normal and transformed cells such as HEK293T, hepatoma, and aortic smooth muscle cells under normoxic conditions (36–39).

Hypoxia enhances the expression of Bnip3, which is regulated mainly at the level of transcription under the control of HIF-1. We have shown that NO-induced Bnip3 expression was also regulated via the hypoxia-responsive element in the Bnip3 promoter and that serial deletion of the promoter constructs and point mutation of binding sites for other NO-responsive transcription factors (p53 and Egr-1) did not affect the activity of the Bnip3
We next showed that Ras activation mediates NO-induced BNIP3 expression. The effect was observed at the level of Bnip3 promoter activation, the induction of BNIP3 protein from Pro-GFP-BNIP3 constructs, and the expression of endogenous protein in RAW264.7 and HEK293T cells (Figs. 4 and 5). The extent of Bnip3 promoter activation by Ras was roughly similar to that achieved by treatment with 100 μM SNAP and was consistently blocked by U0126 or by mutation of the hypoxia-response element in the Bnip3 promoter (Figs. 4C and Fig. 7B), suggesting that Ras and NO are on a common pathway responsible for activating Bnip3 promoter activity. Furthermore, ras-S17N and ras-C118S (a mutant form of Ras that cannot be nitrosylated) blocked NO-induced Bnip3 expression (Fig. 4D), indicating that Ras acts downstream of NO to induce BNIP3 expression. Our findings thus provide evidence that BNIP3 expression is regulated by the NO/Ras/MEK/ERK/HIF-1/Bnip3 signaling pathway. They show, for the first time, that the activated Ras pathway can be responsible, at least in part, for the induction of macrophage death by NO. Although a clear understanding of the signaling pathway of NO-induced apoptosis remains elusive, NO-induced apoptosis is known to be caused by multiple pathways, including p53 induction, mitochondrial stress, and endoplasmic reticulum stress (4, 5, 42). Macrophage apoptosis in response to NO must be the result of a balance of the effects of different pro- and anti-apoptotic factors.

Regulation of the expression of pro-apoptotic molecules by Ras has been shown in various normal and cancer cells. The level of p53 can be regulated by Ras activation through a balance of the opposing effects of Ras-induced Mdm2 and p19ARF (33). Ras-mediated cell growth arrest and apoptosis are associated with cyclin D1 degradation in Rat-1 fibroblasts (35). TRAIL (tumor necrosis factor-related apoptosis-inducing ligand)-induced apoptosis of colon cells is accompanied by up-regulation of death receptors 4 and 5 by Ras (43), and targeted deletion of K-ras protects colon cancer cell lines from 5-fluorouracil- and butyrate-induced apoptosis by inducing gelsolin expression (44, 45). Thus, the components regulated by Ras in each apoptotic pathway seem to be influenced by factors such as cell type, tissue lineage, and the context of the downstream signaling pathway. We found that the levels of p53, cyclin D1, Bad, and Bax expression in RAW264.7 macrophages were not significantly changed by expression of a constitutively active form of Ras, in sharp contrast to the profound enhancement of Bnip3 expression (Fig. 5C). We also showed that silencing Bnip3 expression with an antisense construct almost completely inhibited Ras-induced cell death (Fig. 10B). These observations led us to conclude that Bnip3 is the major downstream effector molecule for Ras-induced death of RAW264.7 cells.

It has been hypothesized that hypoxic induction of BNIP3 early in the development of human carcinomas selects apoptosis-resistant clones within the hypoxic region, leading to more aggressive malignant phenotypes in later stages. However, there are recent reports that down-regulation of BNIP3 expression plays an important role in the progression of various types of cancers. For example, BNIP3 expression is down-regulated in pancreatic cancer, although expression of other hypoxia-responsive genes, including those encoding GLUT1 (glucose...
transporter 1), and IGFBP3 (insulin-like growth factor-binding protein 3), increases (46). Most cases of silencing of BNIP3 expression take place at the transcriptional level as a result of CpG methylation of its promoter (46–48). It has been further reported that the BNIP3 promoter is CpG-methylated in most pancreatic adenocarcinomas (46, 47), in 66% of primary colorectal cancers, in 49% of gastric cancers, and in 10–20% of acute lymphocytic/myelogenous leukemias and multiple myelomas, but not in normal tissues adjacent to the tumors (48, 49). More interestingly, both methylation of the BNIP3 promoter and oncogenic mutation of Ras are most frequently found in pancreatic and colon cancers. Nevertheless, it remains to be determined whether BNIP3 expression is regulated by Ras activation in the various types of cancer cells.

In summary, our results demonstrate that (a) BNIP3 induction by NO is mediated by Ras activation via a MEK/ERK/HIF-1 signaling pathway and that (b) Ras activity leads to macrophage apoptosis by inducing BNIP3. Further study of the regulatory relationship between Ras and BNIP3 in other types of cells will be required for complete understanding of the relationship between Ras and BNIP3 in the induction of apoptosis.

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