Reactive Oxygen Species Produced by NAD(P)H Oxidase Inhibit Apoptosis in Pancreatic Cancer Cells*

Eva C. Vaquero‡, Mouad Edderkaoui‡, Stephen J. Pandol, Ilya Gukovsky, and Anna S. Gukovskaya§

From the Departments of Medicine, Veterans Affairs Greater Los Angeles Healthcare System and UCLA, Los Angeles, California 90073

One reason why pancreatic cancer is so aggressive and unresponsive to treatments is its resistance to apoptosis. We report here that reactive oxygen species (ROS) are a prosurvival, antiapoptotic factor in pancreatic cancer cells. Human pancreatic adenocarcinoma MIA PaCa-2 and PANC-1 cells generated ROS, which was stimulated by growth factors (serum, insulin-like growth factor I, or fibroblast growth factor-2). Growth factors also stimulated membrane NAD(P)H oxidase activity in these cells. Both intracellular ROS and NAD(P)H oxidase activity were inhibited by antioxidants tiron and N-acetylcysteine and the inhibitor of flavoprotein-dependent oxidases, diphenylene iodonium, but not by inhibitors of various other ROS-generating enzymes. Using Rho² cells deficient in mitochondrial DNA, we showed that NAD(P)H oxidase is a major source of growth factor-induced ROS in pancreatic cancer cells. Among proteins that have been implicated in NAD(P)H oxidase activity, MIA PaCa-2 and PANC-1 cells do not express the phagocytic gp91phox subunit but express several nonphagocytic oxidase (NOX) isoforms. Transfection with Nox4 antisense oligonucleotide inhibited NAD(P)H oxidase activity and ROS production in MIA PaCa-2 and PANC-1 cells. Inhibiting ROS with the antioxidants, Nox4 antisense, or MnSOD overexpression all stimulated apoptosis in pancreatic cancer cells as measured by internucleosomal DNA fragmentation, phosphatidylserine externalization, cytochrome c release, and effector caspase activation. The results show that growth factor-induced ROS produced by NAD(P)H oxidase (probably Nox4) protect pancreatic cancer cells from apoptosis. This mechanism may play an important role in pancreatic cancer resistance to treatment and thus represent a novel therapeutic target.

Pancreatic adenocarcinoma is an aggressive malignancy resistant to chemotherapy and radiotherapy (1). One mechanism mediating pancreatic cancer aggressiveness and unresponsiveness to treatment is its resistance to apoptosis. Constitutive activation of antiapoptotic proteins such as transcription factors NF-κB (2) and signal transducers and activators of transcription (3), heat shock proteins (4), or phosphatidylinositide 3-kinase (5) is thought to contribute to pancreatic cancer resistance to apoptosis. We hypothesized that a key factor mediating this resistance is ROS³ generated in pancreatic cancer cells. Although ROS have long been thought to promote cell death (6–8), recent data (9–12) suggest that they may also play a prosurvival role. ROS can activate the above mentioned antiapoptotic signaling pathways. In this regard, we recently showed (13) that compounds with antioxidative properties, quercetin, resveratrol and genistein, stimulated apoptosis of pancreatic cancer cells both in vitro and in vivo.

ROS are highly reactive O₂ metabolites that include superoxide radical (O₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radical (OH·) (14). Large quantities of ROS are produced by phagocytes, mediating host defense against invading microorganisms (15). ROS generation was also detected in nonphagocytic cells (14, 16, 17). Human tumor cells produce significant amounts of ROS, albeit smaller than in phagocytes (18). Sources of cellular ROS include leakage from the mitochondrial electron transport chain as well as a number of ROS-generating plasma membrane and cytosolic enzymes (14). In phagocytes, large amounts of ROS are produced during the oxidative burst by the plasma membrane NAD(P)H oxidase, a well-characterized multicomponent enzyme with gp91phox and p22phox catalytic subunits that together form an integral complex called flavocytochrome b₅₅₈ (15). Recently, proteins of the nonphagocytic oxidase (NOX) family homologous to gp91phox have been shown to generate ROS in nonphagocytic, in particular, cancer, cells (12, 19–21). Furthermore, functional components of the phagocytic NAD(P)H oxidase have been found to mediate superoxide production in some nonphagocytic cells (22, 23). Other ROS-generating enzymes are xanthine oxidase, nitric-oxide synthase (NOS), phospholipase A₂ (PLA₂), and lipooxygenases (14).

A growing body of evidence indicates that ROS play signaling roles in both physiologic and pathophysiologic processes, including proliferation (24), adhesion (25), and hypertension (17, 26). In particular, growth factors are known to stimulate ROS in a variety of cell types through receptor-transducing pathways,

* This work was supported by the Department of Veterans Affairs Merit Review and National Institutes of Health Grant DE-59936 (to A. S. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: VA Greater Los Angeles Healthcare System, West Los Angeles Healthcare Center, Bldg. 258, Rm. 340, 11301 Wilshire Blvd., Los Angeles, CA 90073. Fax: 310-268-4578. E-mail: agukovsk@ucla.edu.

§ These authors contributed equally to this work.

1 The abbreviations used are: ROS, reactive oxygen species; CMX-Ros, Mitotracker Red; DPI, diphenylene iodonium; FACS, fluorescence-activated cell sorter; FBS, fetal bovine serum; FGF-2, basic fibroblast growth factor; IGF-I, insulin-like growth factor-I; MnSOD, manganese superoxide dismutase; mtDNA, mitochondrial DNA; NAC, N-acetylcysteine; AMC, aminoacyl-tRNA synthetase; NOS, nitric-oxide synthase; PLA₂, phospholipase A₂; DCFH-DA, 2',7'-dichlorofluorescin dichloride; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; NOX, nonphagocytic oxidase; ELISA, enzyme-linked immunoassay; t-NAM, N-nitro-L-arginine methyl ester; AAOCPF₃, arachidonoyl trifluoromethylketone.
although the detailed mechanism is poorly understood (14). Growth factor-induced ROS production is believed to be necessary for optimal propagation of mitogenic signals (24). Recent data have demonstrated an important role of ROS in neoplastic proliferation. Nox1 overexpression transforms normal fibroblasts and creates a cell line that is tumorigenic in athymic mice (27, 28). Nox4 was found to regulate growth of malignant melanoma cells (21); Nox5 was found to regulate growth of prostate cancer cells (12).

Compared with their roles in growth and proliferation, the effect of ROS on cell death is less well understood. Most data show that ROS stimulate cell death (6–8). In particular, exogenous H2O2 causes apoptosis at low doses and necrosis at high doses (29). Recent data suggest, however, that ROS inhibit apoptosis in smooth muscle (9), leukemia cells (10), colorectal (11), and prostate cancer cells (12). The prosurvival effect of ROS can be mediated through antiapoptotic redox-sensitive pathways known to be activated by ROS (e.g. NF-κB and heat shock proteins) (30, 31).

Little is known about the sources and the roles of ROS in pancreatic cancer cells. Inhibition of lipoxigenase (a putative source of ROS) as well as overexpression of an antioxidant enzyme, MnSOD, attenuated the growth of human pancreatic cancer cells (32, 33).

The present study sought to determine the sources of ROS generation, their stimulation by growth factors, and whether ROS affect apoptosis in pancreatic cancer cells. We found that growth factors stimulate ROS generation by activating membrane nonmitochondrial NAD(P)H oxidase (probably Nox4) and that inhibiting ROS by different approaches stimulates apoptosis in pancreatic cancer cells. The prosurvival effect of ROS may be an important mechanism of pancreatic cancer cell resistance to therapy.

**EXPERIMENTAL PROCEDURES**

**Reagents—**2′,7′-Dichlorofluorescein diacetate (DCFH-DA) was from Molecular Probes, Inc. (Eugene, OR); Ac-Asp-Glu-Val-Asp-AMC was from Peptide Institute, Inc. (Osaka, Japan); benzoylloxycarbonyl-Val-Ala-Asp(OMe)-CH2F was from Enzyme Systems Products (Livermore, CA). The Nox4 antibody generated from a His-tagged recombinant enzyme, MnSOD, attenuated the growth of human pancreatic cancer cells. Inhibition of lipoxygenase (a putative shaper protein) (30, 31).

**Cell Culture—**Human pancreatic adenocarcinoma cell lines, the poorly differentiated MIA PaCa-2 and moderately differentiated PANC-1, were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in 1:1 Dulbecco's modified Eagle's medium/F-12 medium (Gibco) supplemented with 15% fetal bovine serum (FBS), 4 mM 1-glutamine, and antibiotic/antimicotic solution (Omega Scientific, Tarzana, CA). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO2 and were used between passages 4 and 12. For analyses, cells were plated at a density of 5 × 104/ml in 100-mm culture dishes in the presence and absence of 15% FBS, 100 ng/ml insulin-like growth factor-I (IGF-1), and 50 ng/ml basic fibroblast growth factor (FGF-2) or the indicated inhibitors and cultured for up to 72 h in Dulbecco's modified Eagle's medium/F12 medium.

**Generation of Rho0 Cells—**MIA PaCa-2 Rho0 cells depleted of mitochondrial DNA (mtDNA) were generated by incubating wild type cells (Rho+) for 6–8 weeks with 100 ng/ml ethidium bromide. The medium was supplemented with 4.5 mg/ml glucose, 50 μg/ml uridine, and 100 μg/ml pyruvate to compensate for the respiratory metabolism deficit as previously described (34, 35). After selection, the MIA PaCa-2 Rho0 cells were cultured in the above specified medium without ethidium bromide. To verify the mtDNA depletion, total cellular DNA was extracted and subjected to PCR using two pairs of human mtDNA specific primers: 1) Mts1 (forward) (5′-ctggatgataagcagcactaa) and Mts1a (reverse) (5′-tagagcagacgctggaggg), which gave a 630-bp product, and 2) Mts2 (forward) (5′-aacatccacagctggactgaggtag) and Mts2a (reverse) (5′-ggcagggtagtacagcttggtg), which gave a 532-bp product (36, 37). For control, we measured the expression of β-actin, which is coded by chromosomal DNA.

**Measurement of Intracellular ROS Levels—**Intracellular ROS levels were measured by flow cytometry in cells loaded with the redox-sensitive dye DCFH-DA (38). The fluorescent DCFH-DA readily diffuses into the cells, where it is hydrolyzed to the polar derivative DCFH, which is oxidized in the presence of H2O2 to the highly fluorescentDCF. Approximately 1 × 106 cells were incubated in the dark for 30 min at 37 °C with 10 μM DCFH-DA, harvested, and resuspended in the medium without DCFH-DA. Fluorescence was recorded on FL-1 channel of FACScan® (Becton Dickinson).

**Measurement of NAD(P)H Oxidase Activity—**Superoxide production was measured in total cell homogenates or in membrane and cytosolic fractions by using lucigenin-derived chemiluminescence as described in Refs. 39 and 40. Briefly, 50 μg of protein was diluted in 500 μl of 50 mM phosphate buffer containing 1 mM EGTA and 150 mM sucrose. Dark-adapted lucigenin was added to the sample, and chemiluminescence measurement was immediately started. Chemiluminescence (in arbitrary units) was measured at 15-s intervals for 1 min in a Turner 20/20 luminometer (Turner Designs, Sunnyvale, CA). NADPH or NADH (100 μM each) were used as substrates. The specificity of the measurement was confirmed by adding either a nonenzymatic superoxide scavenger, tiron (10 mM), or superoxide dismutase (200 units/ml; Sigma). In some experiments, the homogenate was preincubated for 30 min with various inhibitors of ROS-generating enzymes as described in the legend of Fig. 3. In this and other assays, protein concentration was measured by the Bradford assay (Bio-Rad).

It has been shown (41, 42) that at high doses lucigenin can by itself stimulate additional superoxide production, which is especially pronounced with NADH as a substrate. This may result in overestimation of NADPH oxidase activity in the homogenate. To exclude the artificial effect of lucigenin, we performed the following experiments. Chemiluminescence was measured in cell homogenates at 5, 25, 50, 100, and 200 μM lucigenin in the presence and absence of NADPH or NADH. At lucigenin concentrations 5–50 μM, the results showed much higher chemiluminescence with NADPH than with NADH. With 100–200 μM lucigenin, NADH-induced signals became larger than those induced by NADPH. The responses obtained with 5–50 μM but not with 100–200 μM lucigenin were blocked by DPI, an inhibitor of NADPH-dependent oxidases. In accord with the data presented in Ref. 42, these results show that 5–50 μM lucigenin does not induce an artificial O2 production in pancreatic cancer cells. The kinetics of the chemiluminescence response was the same with lucigenin concentrations 5–50 μM; however,
the magnitude of the signal was greater at 25 and 50 μM. Thus, the data presented were obtained with 25 or 50 μM lucigenin.

Preparation of Total Cell Lysates—Cells were incubated in a lysis buffer (0.5 mM EDTA, 150 mM NaCl, 50 Tris, 0.5% Nonidet P-40, pH 7.5) for 1 h at 4 °C and centrifuged for 10 min at 13,000 × g, and supernatants were collected and stored at −80 °C until assayed.

Cell Fractionation—Cells were resuspended in a lysis buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1 mM Na-EGTA, 1 mM Na-EDTA, 2 mM MgCl₂, pH 7.0), allowed to swell for 30 min at 4 °C, and then disrupted by 80 strokes in a Dounce homogenizer. Homogenates were centrifuged at 1,000 × g to pellet nuclei and cell debris. Supernatants were centrifuged at 13,000 × g for 30 min, and the cytosolic fraction (supernatant) was collected. The pellet (heavy membranes enriched with mitochondria) was lysed in radioimmune precipitation buffer (0.15 M NaCl, 50 mM Tris, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, pH 7.2) for 1 h. To validate the quality of cytosolic and mitochondrial separation, both fractions were assessed by immunoblotting for the mitochondrial marker cytochrome c oxidase subunit IV.

Reverse Transcription-PCR—The procedures were as we described previously (43, 44). Briefly, total RNA was obtained from pancreatic cancer cells with TRI reagent (Molecular Research Center, Cincinnati, OH) and reverse-transcribed using the SuperScript II preamplification kit (Invitrogen). cDNA derived from 0.5 μg of total RNA was subjected to PCR with human gene-specific, intron-spanning primers that are described in Table I. Target sequences were amplified at 56–58 °C. The reverse transcription-PCR products were all of the expected size (see Fig. 5).

Measurement of Mitochondrial Membrane Potential (ΔΨm)—Changes in ΔΨm were detected with the potential-sensitive probe MitoTracker Red (CMX-Ros; Molecular Probes), as we described previously (45). During the last 30 min of the incubation period, cells were loaded with 10 nM CMX-Ros for 30 min at 37 °C in the dark, washed twice with phosphate-buffered saline, and analyzed on the FACScan® using FL-3.
ROS and Pancreatic Cancer Cell Death

Fig. 2. ROS generation in pancreatic cancer cells is inhibited by antioxidants and by DPI, an inhibitor of NAD(P)H oxidases. MIA PaCa-2 (A and B) and PANC-1 (C) cells were cultured for 72 h (A and C) or for the indicated times (B) with and without 15% FBS, 100 ng/ml IGF-I, or 50 ng/ml FGF-2, in the presence or absence of the inhibitors of various ROS-generating systems: mitochondrial complex I inhibitor rotenone (1 μM), mitochondrial complex III inhibitor antimycin (1 μg/ml), mitochondrial uncoupler fluorocyanide m-chlorophenylhydrazone (FCCP) (50 μM), superoxide scavenger tiron (10 μM), antioxidant NAC (100 μM), flavoprotein-dependent oxidase inhibitor DPI (15 μM), PLA2 inhibitor AACOCF3 (10 μM), xanthine oxidase inhibitor allopurinol (1 μM), phagocytic NAD(P)H oxidase inhibitor apocynin (1 μM), NOS inhibitor L-NAME (1 μM), NAD(P)H:quinone oxidoreductase inhibitor capsaicin (100 μM). Changes in intracellular ROS were measured by FACS analysis in cells labeled with DCFH-DA. DCF fluorescence was normalized to that in cells cultured without inhibitors. B, cells were serum-starved for 72 h, and then FBS with or without inhibitors was added at zero time, and cells were further cultured for the indicated times. Results are representative of two experiments performed in duplicates, which both gave similar results. Values in A and C are means ± S.E. from at least three independent experiments. *, p < 0.05 versus cells without inhibitors.

Transfections—Transient transfection with human MnSOD was performed using the LipofectAMINE 2000 reagent (Invitrogen). The pcDNA3-MnSOD plasmid was kindly provided by Dr. Larry W. Oberley (University of Iowa, Iowa City, IA). Transfection was done according to the manufacturer’s instructions, using 5–8 μg of the plasmid DNA and 20 μl of LipofectAMINE for a 60-mm dish. Transfection efficiency was assessed by co-transfection of a green fluorescent protein plasmid. At 48 or 72 h post-transfection, cell lysates were collected, and the expression of the MnSOD protein was measured by Western blot analysis using a polyclonal anti-MnSOD antibody from Upstate Biotechnology.

Transfection of MIA PaCa-2 and PANC-1 cells with Nox4 antisense oligonucleotide was done as described in Ref. 12. 1.5 nmol of the Nox4 antisense phosphorothioate oligonucleotide, 5’-AGCTTCTCCAGGACAGGCC, was applied for a 60-mm dish. The Nox4 scrambled oligonucleotide, 5’-TGAGGAGGTCTTGTGTCCG, was used as a control. 6 h post-transfection, fresh medium was supplied, and cells were cultured for an additional 48 or 72 h before the specified analyses.

Western Blot Analysis—Proteins from cell lysates were separated by 4–20% SDS-PAGE (Invitrogen) and electrophoretically transferred to nitrocellulose membranes. Nonspecific binding was blocked with 5% milk in Tris-buffered saline (4 mM Tris base, 100 mM NaCl, pH 7.5). Membranes were washed in Tris-buffered saline containing 0.05% Tween 20 (TTBS) and incubated for 2 h with the indicated primary antibodies and then for 1 h with horseradish peroxidase-conjugated secondary antibody. Blots were developed with the Supersignal Chemiluminescent Substrate (Pierce).

Measurements of Apoptosis—Internucleosomal DNA fragmentation was measured by using the Cell Death Detection ELISA™ kit (Roche Applied Science) according to the manufacturer’s instructions. Absorbance values were normalized to cell number.

Phosphatidylserine externalization was analyzed with the annexin-V-FLUOS staining kit from Roche Applied Science as we described before (45). Cells were collected and resuspended at a density of 1 × 10^6 cells in 500 μl of binding buffer containing 2 μl of annexin-V and 1 μl of propidium iodide, incubated in the dark for 30 min at room temperature, and analyzed by flow cytometry.

Effector caspase (DEVDase) activity was measured by a fluorogenic assay in whole cell lysates using Ac-Asp-Glu-Val-Asp-AMC as a substrate, as we described before (45). The assay was done at a density of 1 × 10^6 cells in 500 μl of binding buffer containing 2 μl of annexin-V and 1 μl of propidium iodide, incubated in the dark for 30 min at room temperature, and analyzed by flow cytometry.

Cytochrome c release into the cytosol was assayed by Western blot analysis of cytosolic fractions, as we described previously (45).
Statistical Analysis—Results are expressed as means ± S.E. from at least three independent experiments. Statistical analysis was done using unpaired Student’s t test. The value of p < 0.05 was considered statistically significant.

RESULTS

Growth Factors Stimulate Intracellular ROS Production in MIA PaCa-2 and PANC-1 Pancreatic Carcinoma Cells through Flavoprotein-dependent Oxidase—Using the redox-sensitive fluorescence probe DCFH-DA, we determined that growth factors stimulated ROS production in pancreatic cancer cell lines (Fig. 1). In both MIA PaCa-2 (Fig. 1, A and C–E) and PANC-1 cells (Fig. 1B), intracellular ROS levels were higher in the presence of serum (15% FBS), IGF-I, or FGF-2, whereas lack of growth factors caused time-dependent decrease in ROS (Fig. 1D). Moreover, replenishing growth factors to cells that were serum-starved for 3 days resulted in a marked increase in ROS (Fig. 1E). The effect of added IGF-I to restore ROS in serum-starved MIA PaCa-2 cells was less than that of FBS (Fig. 1E), although both FBS and IGF-I had a similar ability to maintain ROS levels (Fig. 1D). We tested that FBS produced similar increases in ROS with and without heat inactivation, indicating that the effect of FBS was not through complement (data not shown).

We used pharmacologic analysis to determine the sources of intracellular ROS (Fig. 2). Rotenone, an inhibitor of the mitochondrial respiratory chain complex I; antimycin, an inhibitor of complex III; and protonophore fluorocyanide m-chlorophenylhydrazone, a mitochondrial uncoupler, all decreased ROS levels, implicating mitochondria as one source of ROS in MIA PaCa-2 and PANC-1 cells (Fig. 2, A and C). Generation of ROS was inhibited by the superoxide scavenger tiron and the antioxidant N-acetylcysteine (NAC). By contrast, we did not observe ROS inhibition with AACOCF₃, an inhibitor of cytosolic PLA₂; allopurinol, a xanthine oxidase inhibitor; apocynin, an inhibitor of the assembly of phagocytic NAD(P)H oxidase; L-NAME, an inhibitor of NOS; and capsaicin, an inhibitor of NAD(P)H:quinone oxidoreductase. The inhibitors were applied at concentrations reported to completely inhibit their target enzymes (15, 46–49).

Compared with all of the enzyme inhibitors tested, maximal inhibition of ROS in both cell lines was produced by DPI (Fig. 2, A and C), an inhibitor of flavoprotein-dependent oxidases (15). The effect of DPI was comparable with that of tiron, the superoxide scavenger. DPI also delayed and inhibited the ROS production induced by the addition of FBS to serum-starved
cells (Fig. 2B). The inhibitory effect of DPI on ROS (as well as of the other antioxidants) was manifest in all conditions studied, namely in cells cultured with FBS, IGF-I, or FGF-2 or without growth factors (Fig. 2).

These results indicate a flavoprotein-dependent oxidase as a major source of growth factor-stimulated ROS in pancreatic cancer cells. In subsequent experiments, we tested whether this source was indeed NAD(P)H-dependent and whether it was mitochondria-related.

Growth Factors Stimulate NAD(P)H Oxidase Activity in Pancreatic Cancer Cells—In both MIA PaCa-2 and PANC-1 cells, NAD(P)H oxidase activity was present in total cell homogenates and in the membrane but not cytosolic fractions (Fig. 3A). NAD(P)H oxidase activity was stimulated in cells cultured with FBS or IGF-I, and it was almost completely prevented by DPI (Fig. 3, B and C).

Because DPI is a specific inhibitor of flavoproteins, it inhibits phagocytic and nonphagocytic NAD(P)H oxidases, nitric-oxide synthase, NAD(P)H quinone:oxidoreductase, and mitochondrial NADH dehydrogenase (15, 20, 50–52). Fig. 3D shows that whereas NAD(P)H oxidase activity was blocked by DPI, it was not affected by apocynin, an inhibitor of phagocytic NAD(P)H oxidase, or by the inhibitors of other ROS-generating enzymes specified above: allopurinol, AACOCF₃, l-NAME, and capsaicin. These results suggest against the involvement of phagocytic NAD(P)H oxidase, NOS, and NAD(P)H quinone:oxidoreductase (which all use NADPH as a substrate) in ROS generation in pancreatic cancer cells.

To evaluate the contribution of mitochondrial NADH dehydrogenase, we next generated Rho⁰ cells depleted of the mtDNA that codes for this enzyme.

FBS and IGF-I Stimulate ROS in Rho⁰ Cells—MIA PaCa-2 Rho⁰ cells depleted of mtDNA were developed by incubating wild type cells (Rho⁺) for >6 weeks with 100 ng/ml of ethidium bromide. Rho⁰ and Rho⁺ cells were cultured for 72 h with 15% FBS or 100 ng/ml IGF-I. A, PCR analysis demonstrating the mtDNA depletion in Rho⁰ cells. Note the similar levels of β-actin in both Rho⁰ and Rho⁺ cells. B, immunoblot of cytochrome c oxidase I (COX I) showing that this protein, which is coded by mtDNA, is present in Rho⁺ but not in Rho⁰ cells. C, NADPH-dependent superoxide production, as measured by lucigenin-derived chemiluminescence, is preserved in Rho⁰ cells and is abolished by DPI and tiron. D, growth factors stimulate intracellular ROS production in Rho⁰ cells labeled with DCFH-DA. E, superoxide production is increased in homogenates from Rho⁰ cells cultured with growth factors. In D and E, values were normalized to those in cells without growth factors. Values are means ± S.E. from at least four independent experiments. *, p < 0.05 versus cells without growth factors.
in Rho<sup>0</sup> cells (Fig. 4, D and E). These results indicate that the mitochondrial NADH dehydrogenase is not a major contributor to growth factor-induced ROS generation in MIA PaCa-2 cells.

**Multiple NAD(P)H Oxidase Subunits Are Expressed in Pancreatic Cancer Cells, and Nox4 Mediates ROS Production in Both MIA PaCa-2 and PANC-1 Cells**—With reverse transcription-PCR, we assessed the expression of different components of the phagocytic NAD(P)H oxidase complex, as well as of the recently characterized NOX family proteins (19, 20), in pancreatic cancer cells (Fig. 5A). Both MIA PaCa-2 and PANC-1 express mRNAs for Nox4 and (less strongly) Nox5 isoforms. Nox3 is expressed by MIA PaCa-2 but not by PANC-1 cells, whereas neither cell expresses Nox1 or -2. Importantly, the absence of Nox2 (gp91<sup>phox</sup>), the catalytic subunit of neutrophil NAD(P)H oxidase (15), indicates that the “classic” phagocytic NAD(P)H oxidase complex is not present in MIA PaCa-2 and PANC-1 cells. Indeed, neither intracellular ROS nor NAD(P)H oxidase activity in these cells were affected by apocynin, an inhibitor of the phagocytic NAD(P)H oxidase complex assembly (Figs. 2A and 3D).

Both cell lines express p22<sup>phox</sup>, another catalytic subunit of the phagocytic NAD(P)H oxidase complex. Regulatory subunits of NAD(P)H oxidase, p47<sup>phox</sup>, and p67<sup>phox</sup> are expressed in MIA PaCa-2 but not in PANC-1 cells (Fig. 5A). These subunits can mediate ROS generation in cells lacking gp91<sup>phox</sup> through interaction with NOX proteins (20).

We applied the antisense approach to test the role of Nox4 in ROS production. Transfection with Nox4 antisense oligonucleotide resulted in a decrease in the Nox4 protein level in both cell lines (Fig. 5, B and E). In MIA PaCa-2 cells cultured in the presence of FBS, the activity of NAD(P)H oxidase was inhibited ~2-fold by Nox4 antisense, and intracellular ROS level was ~15% lower than in control (transfected with “scrambled” oligonucleotide) cells (Fig. 5, C and D). Transfection of PANC-1 with Nox4 antisense resulted in >2-fold inhibition of intracellular ROS, compared with control (Fig. 5F). Thus, Nox4 is involved in ROS production in both cell lines. Inhibition of intracellular ROS levels with Nox4 antisense oligonucleotide was more pronounced in PANC-1 than in MIA PaCa-2 cells, suggesting that other NOX proteins (e.g., Nox3) contribute to ROS production in MIA PaCa-2 cells.

**Growth Factors Inhibit Internucleosomal DNA Fragmentation in MIA PaCa-2 Cells: Dose Dependences for Growth Factor-induced Inhibition of Apoptosis and Stimulation of ROS Superimpose**—FBS and individual growth factors (IGF-1 and FGF-2) protected MIA PaCa-2 cells from apoptotic death as
measured by internucleosomal DNA fragmentation, a hallmark of apoptosis (Figs. 6 and 7). The dose dependences for growth factor-induced inhibition of DNA fragmentation and stimulation of ROS superimpose (Fig. 6), suggesting that ROS have a prosurvival role in pancreatic cancer cells.

Antioxidants Stimulate Apoptosis in Pancreatic Cancer Cells—To evaluate the involvement of ROS in death responses, we first measured the effects of antioxidants on internucleosomal DNA fragmentation in MIA PaCa-2 and PANC-1 cells. The compounds that inhibited intracellular ROS (tiron, NAC, and DPI) all stimulated DNA fragmentation (Fig. 7, A and B). In contrast, there was no effect on DNA fragmentation with inhibitors that did not decrease the intracellular ROS or NADPH oxidase activity: apocynin, allopurinol, AACOCF₃, L-NAME, and capsaicin (Fig. 7A). Tiron, NAC, and DPI also increased DNA fragmentation in MIA PaCa-2 Rho⁰ cells (Fig. 7C). The stimulation of DNA fragmentation in MIA PaCa-2 Rho⁰ cells by the antioxidants was very pronounced, up to 4-fold.

Importantly, DPI and tiron almost completely abolished the
antiapoptotic effect of serum and the individual growth factors (Fig. 7D). In other words, in the presence of antioxidants, the growth factors did not protect MIA PaCa-2 cells from death, indicating a prosurvival role for ROS.

The effect of antioxidants on DNA fragmentation was time- and dose-dependent. Fig. 8A shows that the decrease in intracellular ROS elicited by DPI precedes the increase in DNA fragmentation; the decrease in ROS was already manifest after 3-h treatment with DPI, whereas DNA fragmentation only increased at later time points. In MIA PaCa-2 (Fig. 8B) and PANC-1 cells (Fig. 8C), the changes in ROS and DNA fragmentation both occurred at DPI concentrations from 2.5 to 10–15 μM.

Because our data showed that Nox4 mediates ROS production in pancreatic cancer cells (Fig. 5), we measured the effect of Nox4 antisense transfection on cell death. We found a significant increase in DNA fragmentation in both MIA PaCa-2 and PANC-1 cells transfected with Nox4 antisense (but not with control) oligonucleotide (Fig. 9, A and B). The stimulation of apoptosis was more pronounced in PANC-1 than in MIA PaCa-2 cells, correlating with greater inhibition of ROS produced by Nox4 antisense in PANC-1 cells (Fig. 5, D and E).

We also overexpressed the superoxide-metabolizing enzyme, MnSOD, in MIA PaCa-2 cells (Fig. 9, C and D), resulting in a decrease in ROS and increased DNA fragmentation (Fig. 9D).

The antioxidants had stimulatory effects not only on DNA fragmentation but also on other parameters of apoptosis in pancreatic cancer cells (Fig. 10). They increased phosphatidylserine externalization as measured by staining with annexin-V and propidium iodine (Fig. 10A). The antioxidants caused cytochrome c release and mitochondrial depolarization (Fig. 10, B and C), indicating the involvement of the mitochondrial pathway of apoptosis. Apoptosis induced by antioxidants is caspase-dependent; it was associated with effector caspase activation (Fig. 10D), and the antioxidants’ effects on both the caspase activation and DNA fragmentation were inhibited by benzoyloxycarbonyl-Val-Ala-Asp(OMe)-CH₂F, a broad-spectrum caspase inhibitor (Fig. 10, D and E).

**DISCUSSION**

The results in the present study show that pancreatic cancer cells produce ROS, which is stimulated by growth factors. Inhibitory analysis indicated that both the mitochondria and nonmitochondrial sources contribute to ROS production. Among the inhibitors of various ROS-generating systems tested, maximal inhibition of ROS in both MIA PaCa-2 and PANC-1 cells was produced by DPI, an inhibitor of flavoprotein-dependent oxidases. We next determined that membrane NAD(P)H oxidase activity is present in pancreatic cancer cells; it was stimulated by FBS and IGF-I and was blocked by DPI.
By contrast, inhibition of xanthine oxidase, NOS, or NAD(P)H quinone oxidoreductase decreased neither intracellular ROS nor NAD(P)H oxidase activity.

In addition to NAD(P)H-dependent oxidases, DPI inhibits the mitochondrial NADH dehydrogenase. Because this enzyme is coded by mitochondrial DNA, we generated mtDNA-deficient Rho0 cells to determine its contribution to growth factor-stimulated ROS production. In MIA PaCa-2 Rho0 cells lacking the mitochondrial NADH dehydrogenase, FBS and IGF-I stimulated intracellular ROS production and NAD(P)H oxidase activity to the same extent (or even greater) than in parental Rho+/H11001 cells. Thus, the mitochondrial NADH dehydrogenase does not contribute much to the growth factor-induced ROS production in pancreatic cancer cells.

In phagocytes, the nonmitochondrial NAD(P)H oxidase is a multicomponent complex that consists of two membrane proteins, gp91phox and p22phox, which comprise the catalytic center of the enzyme (flavocytochrome b558), and four cytosolic factors, p47phox, p67phox, p40phox, and Rac (15). The activation of the enzyme occurs through assembly of the cytosolic factors with flavocytochrome b558. Our data show that pancreatic cancer cells lack gp91phox, the key catalytic subunit of phagocytic NAD(P)H oxidase. The regulatory cytosolic subunits p47phox and p67phox, which are obligatory for phagocytic NAD(P)H oxidase, are expressed in MIA PaCa-2 but not in PANC-1 cells. However, both cell lines express p22phox. These data demonstrate that membrane NAD(P)H oxidase in pancreatic cancer cells is different from the phagocytic enzyme.

Over the past several years, it was shown that gp91phox belongs to a family of homologous proteins, the NOX proteins, which are believed to mediate ROS production in nonphagocytic cells (19, 20). NOX proteins can produce ROS by themselves or in complexes with components of the phagocytic NAD(P)H oxidase (17, 54). We found the expression of Nox4 and Nox5 in both cell lines and Nox3 in MIA PaCa-2. Furthermore, transfection of MIA PaCa-2 cells with Nox4 antisense oligonucleotide decreased cellular ROS levels and NAD(P)H oxidase activity in both MIA PaCa-2 and PANC-1 cells, suggesting that Nox4 mediates ROS production in these cells. Of note, the inhibitory effects of Nox4 antisense (or DPI) on NAD(P)H oxidase activity were much greater than on intracellular ROS levels, indicating that in addition to NOX proteins, other sources contribute significantly to ROS production in pancreatic cancer cells.

Recent evidence indicates an important role for NAD(P)H oxidases in signaling neoplastic proliferation. For example, Nox4 was found to regulate growth of malignant melanoma cells (21); Nox5 mediates growth of prostate cancer cells (12). In contrast to their function in mediating proliferation, the regulatory role of NOX proteins (as well as of ROS in general) in apoptosis is not well understood. Exogenous H2O2 causes apoptosis or necrosis in many cell types, and these data led to a
general belief that ROS promote cell death (6–8). On the other hand, both stimulation (6–8) and inhibition of apoptosis (9–12, 55–57) was reported with antioxidants. In principle, ROS can affect cell death both positively and negatively. ROS may cause cell death (apoptosis or necrosis) by damaging the mitochondria and plasma membrane. However, ROS are also known to activate antiapoptotic pathways such as NF-xB and the heat-shock proteins Hsp70 and Hsp27 (30, 31). Superoxide inhibited FAS-induced apoptosis in a leukemia cell line (10).

In the present study, we found that inhibition of ROS with antioxidants tiron and NAC or with the NAD(P)H oxidase inhibitor, DPI, stimulated apoptosis in pancreatic cancer cells. Furthermore, the antioxidants abrogated the antiapoptotic effect of growth factors (PBS, IGF-I, and FGF-2). Inhibition of ROS with Nox4 antisense or MnSOD overexpression decreased ROS levels and stimulated apoptosis. Thus, ROS generated via the NAD(P)H oxidase-dependent pathway have an antiapoptotic, prosurvival role in pancreatic cancer cells, which is mediated at least in part by Nox4. The prosurvival role for NAD(P)H oxidase was also reported in prostate cancer cells (12).

The detailed mechanism of the antiapoptotic effect of ROS is yet to be determined. We found that inhibition of ROS activated key pathways of apoptosis in pancreatic cancer cells, in particular cytochrome c release and effector caspase activation. The antioxidants stimulated internucleosomal DNA fragmentation and phosphatidylserine externalization.

In conclusion, we showed that a nonmitochondrial membrane NAD(P)H oxidase is a major source of intracellular ROS in pancreatic cancer cells. It is stimulated by PBS, IGF-I, and FGF-2 and is inhibited by DPI. MIA PaCa-2 and PANC-1 cells do not express the key phagocytic catalytic gp91<sup>phox</sup> subunit but express several NOX proteins. Our antisense data indicate, in particular, that Nox4 mediates ROS production in both cell lines. Inhibition of ROS by the antioxidants, Nox4 antisense or MnSOD overexpression all stimulated apoptosis in pancreatic cancer cells. The results indicate that growth factor-stimulated NAD(P)H oxidase (probably Nox4) protects pancreatic cancer cells from apoptosis. This may be an important mechanism contributing to pancreatic cancer resistance to apoptosis. In turn, this enzyme could be targeted to stimulate pancreatic cancer cell death.

Acknowledgments—We thank Mohammad Shahsahebi and Yoon Jung for help in preparing the manuscript.

REFERENCES
1. Wanebo, H. J., and Vezeridis, M. P. (1996) Cancer 78, 580–591
2. Aggarwal, B. B. (2000) Biochem. Pharmacol. 60, 1033–1039
3. Battle, T. E., and Frank, D. A. (2002) Curr. Mol. Med. 2, 381–392
4. Beere, H. M., and Green, D. R. (2001) Trends Cell Biol. 11, 6–10
5. Nicholson, K. M., and Anderson, N. G. (2002) Cell Signal. 14, 381–385
6. Simon, H. U., Haj-Yehia, A., and Levi-Schaffer, F. (2000) Apoptosis 5, 415–418
7. Kaminan, K., and Jain, S. K. (2000) Pathophysiology 7, 123–163
8. Davis, W., Jr., Ronai, Z., and Tew, K. D. (2001) J. Pharmacol. Exp. Ther. 296, 1–6
9. Wedgeood, S., and Black, S. M. (2005) Am. J. Physiol. 285, L305–L312
10. Clement, M. V., and Stamenskovic, I. (1996) EMBO J. 15, 216–225
11. Lin, K. I., Pasinelli, P., Brown, R. H., Hardwick, J. M., and Ratan, R. R. (1999) J. Biol. Chem. 274, 13650–13655
12. Brar, S. S., Corbin, Z., Kennedy, T. P., Hemendinger, R., Thornton, L., Bommarius, B., Arnold, R. S., Whorton, A. R., Sturrock, A. B., Hueckstadt, T. P., Quinn, M. T., Krenitsky, K., Artie, R. G., Lambeth, J. D., and Hoidal, J. R. (2003) Am. J. Physiol. 285, C363–C369
13. Mouria, M., Gukovskaya, A. S., Jung, Y., Buckeler, P., Hines, O. J., Reher, H. A., and Pandol, S. J. (2002) Int. J. Cancer 98, 761–769
14. Thannickal, V. J., and Fanburg, B. L. (2000) Am. J. Physiol. 278, L1005–L1028
15. Babior, B. M., Lambeth, J. D., and Nauseef, W. (2002) Arch. Biochem. Biophys. 397, 342–344
16. Li, J. M., and Shah, A. M. (2002) J. Biol. Chem. 277, 19952–19960
17. Lassegue, B., and Clemps, R. E. (2003) Am. J. Physiol. 285, R277–R297
18. Szatrowski, T. P., and Nathan, C. F. (1991) Cancer Res. 51, 784–798
19. Lambeth, J. D. (2002) Curr. Opin. Hematol. 9, 11–17
20. Bokoch, G. M., and Knaus, U. G. (2003) Trends Biochem. Sci. 28, 502–508
21. Brar, S. S., Kennedy, T. P., Sturrock, A. B., Huecksteadt, T. P., Quinn, M. T., Whorton, A. R., and Hosital, J. R. (2002) Am. J. Physiol. 283, C1212–C1224
22. Cheng, G., Cao, Z., Xu, X., van Meir, E. G., and Lambeth, J. D. (2001) Gene 269, 131–140
23. Takeya, R., Ueno, N., Kami, K., Taura, M., Kohjima, M., Izaki, T., Nunei, H., and Sumimoto, H. (2003) J. Biol. Chem. 278, 25234–25246
24. Sauer, H., Wartenberg, M., and Hescheler, J. (2001) Cell Physiol. Biochem. 11, 173–186
25. Chiarugi, P., Pani, G., Giannoni, E., Taddei, L., Colavitti, R., Raugei, G., Symons, M., Borrello, S., Galeotti, T., and Rampini, G. (2003) J. Cell Biol. 161, 933–944
26. Li, J. M., and Shah, A. M. (2003) J. Am. Soc. Nephrol. 14, S221–S226
27. Suh, Y. A., Arnold, R. S., Lassegue, B., Shi, J., Xu, X., Sorensa, D., Chung, A. B., Griendling, K. K., and Lambeth, J. D. (1999) Nature 401, 79–82
28. Arnold, R. S., Shi, J., Murad, E., Whalen, A. M., Sun, C. Q., Polavarapu, R., Parthsarathy, S., Peters, J. A., and Lambeth, J. D. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 5550–5555
29. Sakamoto, T., Repasky, W. T., Uchida, K., Hirata, A., and Hirata, F. (1996) Biochem. Biophys. Res. Commun. 220, 643–647
30. Green, D. R. (2003) Mol. Cell 11, 551–552
31. Garrido, C., Gurbuxani, S., Ravagnan, L., and Kromer, G. (2001) Biochem. Biophys. Res. Commun. 286, 433–442
32. Tong, W. G., Ding, X. Z., Witt, R. C., and Adrian, T. E. (2002) Mol. Cancer Ther. 1, 929–935
33. Cullen, J. J., Weydart, C., Hinkhouse, M. M., Ritchie, J., Demann, F. E., Spitz, D., and Oberley, L. W. (2003) Cancer Res. 63, 1297–1303
34. King, M. P., and Attardi, G. (1989) Science 246, 500–503
35. King, M. P. (1996) Methods Enzymol. 264, 339–344
36. Hail, N., Jr., Youssef, E. M., and Lotan, R. (2001) Cancer Res. 61, 6698–6702
37. Park, K. S., Nam, K. J., Kim, J. W., Lee, Y. B., Han, C. Y., Jeong, J. K., Lee, H. K., and Pak, Y. K. (2001) Am. J. Physiol. 280, E1097–E1104
38. Royall, J. A., and Ischiropoulos, H. (1993) Arch. Biochem. Biophys. 302, 348–353
39. Rajagopalan, S., Kurz, S., Munzel, T., Tarpey, M., Freeman, B. A., Griendling, K. K., and Harrison, D. G. (2001) Free Radic. Biol. Med. 30, 603–612
40. Li, Y., Zhu, H., Kuppusamy, P., Roubaud, V., Zweier, J. L., and Trush, M. A. (1998) J. Biol. Chem. 273, 2015–2023
41. Janiszewski, M., Souza, H. P., Liu, X., Pedro, M. A., Zweier, J. L., and LaRonde, F. R. (2002) Free Radic. Biol. Med. 34, 446–453
42. Gukovsky, I., Gukovskaya, A. S., Blinman, T. A., Zaninovic, V., and Pandol, S. J. (1998) Am. J. Physiol. 275, G1402–G1414
43. Blinman, T. A., Gukovsky, I., Mouria, M., Zaninovic, V., Livingston, K., Pandol, S. J., and Gukovskaya, A. S. (2000) Am. J. Physiol. 279, C1993–C2003
44. Yagi, T., and Matsuno-Yagi, A. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 2548–2550
45. Kolk, M., Rodriguez de Turco, E. B., Diemer, N. H., and Bazar, N. G. (2003) Neurosci. Lett. 338, 164–168
46. Steik, J., Hittermann, T. J., Dijkstra, J. H., and Verhoeven, A. J. (1994) Am. J. Respir. Cell Mol. Biol. 11, 95–102
47. Yagi, T. (1999) Arch. Biochem. Biophys. 361, 305–311
48. Royall, J. A., Ischiropoulos, H., and Butler, A. J. (1998) Biochem. Biophys. Res. Commun. 253, 295–299
49. Rand, M. J., and Li, C. G. (1998) J. Bioenerg. Biomembr. 30, 185–189
50. Yagi, T., and Matsuno-Yagi, A. (2003) Biochemistry 42, 2266–2274
51. Taanman, J. W. (1997) J. Bioenerg. Biomembr. 29, 151–163
52. Yagi, T., and Matsuno-Yagi, A. (2003) J. Biol. Chem. 278, 3510–3513
53. Moreno-Manzano, V., Ishikawa, Y., Lucio-Caraza, J., and Kitamura, M. (2000) J. Biol. Chem. 275, 12864–12891
54. Wen, J., You, K. R., Lee, S. Y., Song, C. H., and Kim, D. G. (2002) J. Biol. Chem. 278, 38954–38964
55. Zhang, X., Shan, P., Sasidhar, M., Chupp, G. L., Flavell, R. A., Choi, A. M., and Lee, P. J. (2003) Am. J. Respir. Cell Mol. Biol. 29, 355–358
Reactive Oxygen Species Produced by NAD(P)H Oxidase Inhibit Apoptosis in Pancreatic Cancer Cells
Eva C. Vaquero, Mouad Edderkaoui, Stephen J. Pandol, Ilya Gukovsky and Anna S. Gukovskaya

J. Biol. Chem. 2004, 279:34643-34654.
doi: 10.1074/jbc.M400078200 originally published online May 23, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M400078200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 57 references, 16 of which can be accessed free at http://www.jbc.org/content/279/33/34643.full.html#ref-list-1