Proline Oxidase, Encoded by p53-induced Gene-6, Catalyzes the Generation of Proline-dependent Reactive Oxygen Species

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Abstract

The p53-dependent initiation of apoptosis is accompanied by the induction of proline oxidase (POX), a mitochondrial enzyme catalyzing the conversion of proline to pyrroline-5-carboxylate with the concomitant transfer of electrons to cytochrome c. However, the contribution of increased POX activity to apoptosis, if any, remains unknown. Using Adriamycin to initiate p53-dependent apoptosis, we showed that the expression of POX is up-regulated in a time- and dose-dependent manner in a human colon cancer cell line (LoVo). In cells expressing POX, the addition of proline increases reactive oxygen species (ROS) generation in a concentration-dependent manner; glutamate, a downstream product of proline oxidation, had no effect. Induction of POX was dependent on the p53 status of the cell. In the conditionally immortalized murine colonic epithelial cell line YAMC, where the p53 phenotype can be modulated by temperature, proline oxidase expression and ROS production could only be induced when the cells were phenotypically p53-positive. To confirm that the observed ROS production was not secondary to some other effect of p53, we also conditionally expressed POX in a p53-negative colon cancer line. Again, we found a proline-dependent ROS increase with POX expression. We hypothesize that proline oxidation supports the generation of ROS by donating reducing potential to an electron transport chain altered either by p53-dependent mechanisms or by overexpression of POX.

Introduction

The importance of ROS in intracellular signaling is increasingly recognized (1). Recent studies have shown that diverse stimuli can increase intracellular oxygen radicals and contribute to signaling events leading to proliferation on the one hand and to cell cycle arrest on the other (2). Importantly, the generation of ROS may be a necessary event in the induction of apoptosis (3, 4). However, the mechanisms mediating the generation, transmission, and targeting of ROS signals in cell regulation and in apoptosis, in particular, are poorly understood.

Using a cultured colorectal cancer cell line (DLD-1), Polyak et al. (5) showed that p53-dependent apoptosis is preceded by the induction (>10-fold) of 14 PIGs of 7720 genes monitored by Serial Analysis of Gene Expression. Several of these PIGs are involved in redox regulation, including PIG6, which encodes POX (6, 7), a mitochondrial inner-membrane enzyme that catalyzes the conversion of proline to P5C. The cytoxic agent Adriamycin also induced POX expression and apoptosis in a p53-positive cell line (LoVo) in a manner similar to that induced by adenovirus-mediated p53 induction in DLD-1 cells (5). The role of POX in this p53-dependent apoptosis, however, remains unknown.

We considered that the contribution of POX to p53-dependent apoptosis is in the realm of perturbation of redox status. The interconversion of proline and P5C forms a metabolic shuttle of redox equivalents between cytosol and mitochondria (8, 9). P5C can be transported into cells as a source of oxidizing potential where its reduction to proline generates NADPH. Proline can then be transported into mitochondria where POX mediates its conversion back to P5C with concomitant production of ATP, thereby completing a proline cycle (10). Physiological effects of proline metabolism have been described in a number of organisms. For example, Drosophila melanogaster sluggish A mutants with POX mutations are defective in initiating movement and phototaxis (11). In plants, proline metabolism plays an important role in stress tolerance and osmotic balance (12), and in cultured cells, P5C and the proline cycle generate oxidizing potential to activate the pentose phosphate shunt and increase nucleotide synthesis by both salvage and de novo pathways (8, 13, 14). In this report, we describe our studies showing the generation of proline-mediated ROS. Whether this metabolic effect plays a necessary or sufficient role in apoptosis remains unknown, but it is tempting to speculate that it may act as a mediator of the p53-induced apoptotic cascade.

Because Adriamycin not only induces POX but also can serve as a substrate for redox cycles (15), we sought a system to dissociate Adriamycin from the process for ROS generation. Furthermore, we asked whether p53 was necessary for the generation of proline-dependent ROS as well as for the induction of POX. Using a p53-negative colorectal carcinoma cell line stably transfected with an inducible POX expression vector, we showed that proline-dependent ROS generation was dependent on POX and independent of Adriamycin treatment. Moreover, in these transfected cells expressing POX, ROS generation appeared independent of p53.

On the basis of these findings, we propose that p53-dependent apoptosis not only is preceded by the induction of POX but also is accompanied by proline-mediated ROS generation. Because it is becoming clear that proline cycling dramatically affects cellular energetics and physiological processes, it is intriguing to search for a more general role for proline in controlling cellular growth; therefore, the role of proline-mediated ROS generation in activating the apoptotic cascade is being actively investigated.

Materials and Methods

Synthesis of PSC. DL-PSC dinitrophenylhydrazone (Sigma, St. Louis, MO) was hydrolyzed to DL-PSC according to the method of Mezl and Knox (16). Free DL-PSC was purified further by cation exchange chromatography, and the concentration was determined colorimetrically using O-aminobenzaldehyde (Sigma, St. Louis, MO).
Cell Culture. The LoVo cell line was obtained from American Type Culture Collection (Rockville, MD) and cultured in the recommended medium at 37°C and 5% CO₂. For POX induction, cells were refed with DMEM (Biofluids, Inc., Rockville, MD) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), penicillin and streptomycin, 2 mM glutamine, and with or without Adriamycin (0.5 μM) for 48 h. YAMC cells (17) were grown in a 75-cm² culture flask coated with type I collagen (5 μg/cm²; Collaborative Biomedical Products, Bedford, MA) in RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 5% neonatal calf serum (GemiBio-Products, Calabasas, CA), ITS (6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenous acid, 5.35 mg/ml lipoic acid, and 1.25 mg/ml BSA; Collaborative Biomedical Products), 5 IU/ml murine IFN-γ (Life Technologies, Inc.), penicillin and streptomycin. These cells were cultured under transformed conditions in a 33°C incubator with 5% CO₂, with all of the aforementioned supplements in the medium. All cells were then transferred, upon attaining confluence, into a 39°C incubator under nontransforming (nonpermissive) conditions in serum-free and IFN-γ-free media for 72 h before each experiment. Adriamycin was used at a concentration of 0.25 μM for these cells.

DLD1-POX Cells. Using a colon cancer tetracycline-inducible system described previously (18), a stable POX-inducible cell line was generated. In brief, a tet-responsive POX expression construct was made by cloning a 2.2-kb restriction fragment containing full-length cDNA of the human POX gene into pBI-MCS-EGFP between the PvuII and Nhel sites. The resulting plasmid pB1-ePOX was linearized with Ase I and cotransfected into DLD1 cells with linearized pTK-hygro (Clontech, Palo Alto, CA) at a molar ratio of 5:1. Single colonies were obtained by limiting dilution with 400 pg/μl of G418 (Geneticin, Life Technologies, Inc., Grand Island, NY) and 250 μg/ml Hygromycin B (Calbiochem, La Jolla, CA) in the presence of 20 ng/ml doxycycline (Sigma) for 3–4 weeks. Clones were selected on the basis of GFP induction, as determined by fluorescence microscopy, in media with and without doxycycline. Clones expressing low but uniform GFP levels upon induction were selected and maintained in McCoy's 5A medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (HyClone Laboratories), 100 units/ml of penicillin, and 100 μg/ml streptomycin in the presence of 0.4 mg/ml G418, 0.25 mg/ml Hygromycin B, and 20 ng/ml doxycycline. Transfections were carried out with FuGENE 6 (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions. DLD1-POX clones were analyzed further for the expression of POX by enzyme assay.

ROS Generation. Cells were cultured in chambered coverglass slides (Nalge Nunc, Napersville, IL) in the respective growth medium for 24–48 h before treatment. The YAMC cells were pretreated under serum-free conditions as mentioned above. Cells were then treated with Adriamycin, proline, glutamate, and/or PSC, as specified, for an additional 6–48 h before analysis for ROS. For inhibition studies, cells were treated with 5 mM N-acetyl cysteine 24 h before and concurrent with Adriamycin/proline treatment.

Measurement of ROS. On the day of the experiment, treatment medium was removed and the monolayer was exposed to serum-free, phenol red-free medium containing 50 mM DCF (Molecular Probes, Inc., Eugene, OR), a dye that will fluoresce when exposed to hydrogen peroxide or hydroxyl radicals (19). Cells were exposed to the dye for 20 min to allow for equilibration, which then was replaced with DCF-free PBS. The fluorescence intensity was determined on an adherent cell laser cytometer (ACAS; Meridian Instruments, Inc. Okemos, MI) using 488 nm excitation and 560 nm fluorescence detection. Quantitation was based on the analysis of fluorescence per cell or per cell area.

Expression of POX by RT-PCR. Total RNA was prepared from harvested cells using Trizol (Life Technologies, Inc.) and quantified using a Beckman DU-65 spectro-photometer. A two-step RT-PCR reaction (0.5 μg of total RNA, 0.5 μg of random primers, and 0.2 μl specific primers in a 50-μl volume) was performed using RT-PCR beads (Pharmacia Biotech, Piscataway, NJ). The reaction mixture was incubated at 42°C for 25 min. POX: Specific oligomers unique to human and mouse POX (forward, 5′ GCT ATT AAG CTC ACA GCA CGT GG 3′; human reverse, 5′ CTG ATG GCC GGC TGG AAG TAG 3′; and murine reverse, 5′ CTG ATG GCC GGC TGG AAG TAG 3′) were designed to amplify a product of 478 bp. The PCR conditions consisted of 29 cycles at 94°C for 45 s at 61°C for 45 s, and at 72°C for 2 min. A 72°C extension for 7 min was added as the final step. The glyceraldehyde 3-phosphate dehydrogenase control primers (Clontech) were used in a reaction with identical conditions, except that the reaction continued for 19 cycles. All reaction products (10 μl with glycerol loading buffer) were run on a 2.5% agarose gel, stained with ethidium bromide, and the products were recorded and quantified using the Electrophoresis Documentation and Analysis System (Kodak Digital Science, Rochester, NY).

Expression of Other Proline-Metabolic Enzymes. We used published methods to perform Northern blots to ascertain the expression of mRNAs for ornithine aminotransferase (20), PSC dehydrogenase (21), and PSC reductase (22).

POX Assay. LoVo cells were grown in the appropriate medium for POX induction after which cells were rinsed and scraped in cold PBS, pelleted at 480 × g, and resuspended in cold sucrose buffer [0.250 M sucrose, 3.5 mM Tris, and 1 mM EDTA (pH 7.4)]. Suspensions were then sonicated for 20 s at a setting of 25% (Branson Sonifier 450; Branson Ultrasonics Corp., Danbury, CT). Total protein was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Reactions were performed using a radioisotopic method to detect 14C-PSC as described previously (23).

Western Blotting. Cell lysates were prepared and quantified according to established methods. To each well of a 7.5% SDS-polyacrylamide gel, 15–30 μg total protein was applied, electrophoresed, and transferred to nitrocellulose membrane using a semi-dry blotter (Bio-Rad). Membranes were blocked using Tris-buffered saline with 3% nonfat milk (pH 8.0; Sigma). Blots were then probed with the primary antibody anti-p21 (1:2000; Transduction Laboratories, Lexington, KY), in blocking buffer, and subsequently by a secondary anti-mouse IgG antibody conjugated to horseradish peroxidase (1:2000). All blots were washed in Tris-buffered saline with Tween 20 (pH 8.0; Sigma). Detection was done using an ECL kit (Amersham, Arlington Heights, IL). Blots were routinely stripped by Encore Blot Stripping Kit (Novus Molecular, Inc., San Diego, CA) and reprobed with anti-actin monoclonal antibody (1:2000) to serve as loading controls.

Results

Adriamycin Induces POX Expression in LoVo Cells. To characterize the induction of POX, LoVo cells were exposed to Adriamycin at various concentrations after which RNA was harvested and analyzed by RT-PCR. Our results indicated that POX expression increased sharply (4-fold) with 0.1 μM Adriamycin after 18 h of exposure. Additional increases in drug concentration resulted in more modest increases in POX expression (Fig. 1A). To assess the time course of POX mRNA expression in response to Adriamycin, we performed studies using 0.5 μM Adriamycin and collected samples of cells exposed to the drug over a 40-h period. RT-PCR analysis indicated that POX expression was induced within 12 h and was maintained at elevated levels throughout the period of exposure to Adriamycin (Fig. 1B). These results are in agreement with previous work showing POX induction to be relatively late in comparison with some other PIGs (5).

POX Enzyme Activity Correlates with Adriamycin-induced POX mRNA Expression in LoVo Cells. To determine whether Adriamycin-induced POX mRNA expression results in increased POX activity, we used a cell-free radioisotopic assay that recovers radio-labeled PSC produced from precursor 14C-proline (23). After 48 h of POX induction by Adriamycin, LoVo cells were harvested and lysates were added to the assay and incubated over a 90-min period. POX activity was found to be 2- to 3-fold higher in the drug-treated cells and remained nearly linear throughout this period (Fig. 1C). Thus, mRNA induction correlates with increased POX enzyme activity in these treated cells.

Adriamycin Induces POX Expression in the Conditionally Immortalized Cell Line YAMC. POX was found to be a member of the p53-inducible gene set in a sage analysis and in a cultured human colon cancer cell line (5). To confirm that this is a generalized effect of p53, we used the conditionally immortalized YAMC cell line to examine the dependence of POX expression on p53 induction. This line was isolated from the colonic epithelium...
of a temperature-sensitive SV40 LT transgenic mouse, permitting modulation of the p53 phenotype (17). Under transforming conditions (permissive temperature, 33°C) T antigen binds p53, allowing unregulated growth of the cell line. However, under nontransforming conditions (nonpermissive temperature, 39°C) T antigen is unable to bind p53, and YAMC cells undergo growth arrest. When we cultured YAMC cells under nontransforming conditions, we found Adriamycin treatment increases POX expression as in the p53-positive LoVo cell line as determined by RT-PCR. In contrast, POX is not induced by Adriamycin in YAMC cells grown at the permissive temperature (Fig. 2A).

To confirm the expected p53 activity in these experiments, we prepared whole cell lysates of YAMC for immunoblot analysis of p21/WAF1. As shown in Fig. 2B, when we cultured the cells under nontransforming conditions (T antigen nonfunctional), Adriamycin induced p21 protein. By contrast, at the permissive temperature, when p53 is inactive, Adriamycin failed to up-regulate p21 (Fig. 2B). These observations demonstrate that YAMC cells conform to the model for p53 functional activity and that p53 is required for up-regulation of POX.

Expression of Other Proline Metabolic Genes. In an effort to understand more fully the effects of Adriamycin and p53 induction on the metabolism of proline, we performed Northern blots using total RNAs from Adriamycin-treated LoVo cells and cDNA probes for proline metabolic enzymes, P5C reductase, P5C dehydrogenase, and ornithine aminotransferase. Interestingly, no other proline-metabolizing genes are induced with p53 induction. However, it is noteworthy that the expression of P5C reductase, the enzyme catalyzing the conversion of P5C back to proline, is reduced to 50% of control levels during the course of Adriamycin-induced POX.

Proline-mediated ROS Generation. Because proline oxidation can affect cellular energetics (10, 24, 25), it is possible that proline-dependent electron transfers may contribute to ROS production. Using Adherent Cell Analysis and Sorting with DCF, we detected increases in ROS production in LoVo cells treated with Adriamycin and proline over treatment with Adriamycin alone. Furthermore, this effect of proline was dose-dependent (Fig. 3A). Without Adriamycin treatment, proline had no effect on ROS generation indicating the requirement for POX induction. Neither glutamate (Fig. 3A) nor P5C (not shown) increased ROS production in Adriamycin-treated cells, indicating that the products of proline catabolism were not responsible for the ROS stimulation. Furthermore, when cells were pretreated with N-acetyl cysteine, a powerful antioxidant, the effects of proline in Adriamycin-treated cells were abolished (data not shown).

Because Adriamycin induced POX in YAMC under conditions where these cells expressed a p53-positive phenotype, we asked whether proline could affect ROS production in YAMC cells. We incubated YAMC cells under nontransforming conditions (nonpermissive temperature, 39°C), induced POX with Adriamycin, and

![Fig. 1. POX expression profiles in Adriamycin-treated LoVo cells. Cells were grown in 25- or 75-cm² flasks to 60–70% confluence as described in “Materials and Methods.” All cells were harvested after a cold PBS rinse, scraped into cold PBS, pelleted, then resuspended in Trizol solution for RNA extraction. RT-PCR, gel electrophoresis, and analysis were performed as described. A, concentration-dependent effects of Adriamycin on POX. Cells were exposed to medium containing different concentrations of the drug for 48 h. B, time course of Adriamycin-induced POX expression. Cells were treated with 0.5 μM Adriamycin and collected at various times. C, POX activity was assessed (28) in LoVo cells after a 48-h treatment with 0.5 μM Adriamycin. Control (□), Adriamycin (△). Data shown are averages from at least three separate determinations. Bars, SD.](image)

![Fig. 2. POX expression profiles in YAMC cells at the permissive (33°C) and nonpermissive (39°C) temperatures. A, YAMC cells were grown to confluence at 33°C as described in “Materials and Methods.” They were then shifted to serum-free media and maintained at 39°C for 2 days, after which cells were either kept at 39°C or placed back at 33°C for 6 h and then given 24 h of exposure to 0.25 μM Adriamycin in serum-free medium or serum-free control medium. RNA was harvested by the Trizol method, and RT-PCR was performed as described previously. B, Western blot analysis of YAMC cells stimulated at the permissive (33°C) or nonpermissive (39°C) temperature after a period of growth at 39°C. Cells were stimulated with 0.25 μM Adriamycin (ADR) or untreated (CON), in duplicate, for 24 h, after which cells were harvested, and whole cell lysates were prepared as described in “Materials and Methods.” Anti-p21, anti-actin, and goat antirabbit horseradish peroxidase-linked IgG were used at dilutions as suggested by the manufacturer.](image)
responded to proline in a dose-dependent fashion, where 1 mM proline more sensitive to proline treatment than the LoVo cells. YAMC cells added increasing amounts of proline. Interestingly, these cells were resulted in a.

Next, we questioned whether culture conditions that limit POX expression had any effect on ROS generation. We cultured YAMC cells under transforming conditions (permissive temperature, 33°C) with Adriamycin and proline. Under conditions where POX is not induced by Adriamycin, proline had no effect on ROS generation. Moreover, proline or Adriamycin alone did not increase ROS above control levels (Fig. 3B). Taken together, these data indicate that in this cell model, POX is required for proline-dependent ROS production.

**POX Transfection of DLD-1 Cells Confers Inducibility of POX Activity and ROS Production.** The use of Adriamycin to induce POX and ROS may introduce ambiguities in that Adriamycin itself is a redox cycling agent capable of mediating electron transfer and ROS production (15). To confirm our interpretation, we used a mutant p53 colon carcinoma cell line DLD-1 stably transfected with human POX cDNA under a tetracycline-regulated promoter in which removal of the tetracycline block allows expression of POX. These cells were cultured for a period with the tetracycline block, then medium without tetracycline was substituted, and POX was induced over a 2-day period. Cellular extracts were prepared and analyzed for POX enzyme activity. Results indicated that POX activity increased 2- to 3-fold over controls in DLD1-POX cells when the promoter is activated (Fig. 4A). ROS production was also analyzed in these cells. Again, after 2 days of promoter activation and increasing proline concentration, ROS levels were elevated up to 2-fold over the induced DLD1-POX controls (Fig. 4B). These results indicate that in the absence of Adriamycin, POX overexpression and proline are sufficient for ROS generation.

**Discussion**

p53-dependent apoptosis is a highly regulated process that is directed by various modifications of the p53 protein that result in multiple levels of control. In response to genotoxic stress, p53 disassociates from its negative regulator, MDM2, and is phosphorylated and acetylated at different positions to promote interactions with other proteins or regulatory elements of target genes involved in cell cycle arrest, apoptosis, or cell survival (26). In general, the set of PIGs encode proteins that generate or respond to oxidative stress (5, 18). Modulation of these sources and quenching of the resulting oxygen-free radicals by PIGs could enable the cell to control a delicate balance of ROS to ensure the appropriate biological response. Although, the downstream effects of ROS production are not well understood, it is clear that ROS does play a role in the mechanisms of cell death, because blocking its production inhibits apoptosis (3, 4).

Of the PIGs with activities related to the redox state of the cell, it is not clear which is the main source of ROS, but it is likely that a number of the PIGs contribute to free radical production and control. ROS production is apparent in cells undergoing p53-dependent apoptosis, but, to our knowledge, none of the PIGs has been shown to directly generate ROS in situ. In an effort to identify contributors to free radical production and apoptosis, expression studies have focused on *PIG3* in that it is related to plant and animal NADPH oxidoreductases. However, overexpression of *PIG3* was not sufficient to induce...
apo9ptosis (5). Our studies are the first to identify POX activity (PIG6) as a source of measurable ROS production that can contribute to the redox state of the cell. Importantly, we found that the expression of POX is not sufficient to produce ROS; the addition of proline substrate is required. This result indicates that the ROS burst is a consequence of POX activity and not attributable to a concomitant effect of POX expression. In addition, downstream products of proline oxidation, i.e., P5C or glutamate, did not significantly or consistently alter ROS production. Proline can be supplied, in vivo, by endogenous synthesis or by the degradation of cellular matrix and the release of proline through dipeptidyl prolidase activity (27). In either situation, it is our belief that p53-induced POX activity and substrate availability make available ROS, which in turn could be regulated by other cellular mechanisms to contribute to an apoptotic end point.

Although the proline-dependent generation of ROS required the expression of POX, the contribution of other p53-dependent mechanisms to this phenomenon remains a likely possibility. However, in p53-negative DLD-1 cells transfected with POX, the generation of ROS required only the induction of POX. Although this finding suggests that POX is sufficient for the generation of ROS, such an interpretation must be made with caution. Metabolic activity catalyzed by the overexpressed enzyme could include aberrant electron transfers, which has been suggested as an ROS-generating mechanism (28). Additional studies defining the subcellular localization of the overexpressed POX protein as well as the energetics of its metabolic activity may shed light on the generation of ROS in these p53-negative cells.

The significance of proline metabolism in apoptosis and carcinogenesis remains to be defined. However, it is clear that the oxidation of this amino acid can contribute to the energy supply of the cell and enhance the generation of ROS, the importance of which is increasingly recognized in the apoptotic response. This alternate electron donor may be critical in supporting an apoptotic paradigm by providing the required ROS. Under normal metabolic situations where the electron transport chain remains intact, POX most likely donates electrons to cytochrome c through an intervening flavoprotein, because downstream inhibitors of electron transport or removal of cytochrome c will block proline oxidation (25). However, with apoptosis, modifications could be made to the electron flow that enable POX activity to support this end point. For example, the functional linkage of POX to cytochrome c in the electron transport chain may be considered an important control because unlinking of these two elements during apoptosis, when cytochrome c is released from mitochondria, could alter electron flow to support ROS production. In fact, completed work by members of our laboratories demonstrates that POX overexpression is accompanied by the hallmarks of apoptosis, including cytochrome c release and caspase activation.4 Additional research defining the role of proline metabolism and the mechanisms involving ROS production is underway.

Acknowledgments

We thank Dr. Bert Vogelstein of the Johns Hopkins Oncology Center, Baltimore, MD, for his advice and help in the generation of the DLD1-POX cells.

References


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*Cancer Res* 2001;61:1810-1815.

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