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Overexpression of proline oxidase induces proline-dependent and mitochondria-mediated apoptosis

Chien-an A. Hu,[1,*] Steven P. Donald,[2,*] Jian Yu,[3] Wei-Wen Lin,[4] Zhihe Liu,[1] Gary Steel,[5] Cassandra Obie,[5] David Valle[5] and James M. Phang[2]

[1]*Department of Biochemistry and Molecular Biology, University of New Mexico School of Medicine, Albuquerque, NM, USA;* [2]*Metabolism and Cancer Susceptibility Section, Basic Research Laboratory, National Cancer Institute at Frederick, 1050 Boyles Street, Bldg 538, Room 115, NCI at Frederick, Frederick, MD, 20895, USA;* [3]*Cancer Institute, University of Pittsburgh, Pittsburgh, USA;* [4]*Department of Psychiatry, Tri-Service General Hospital and National Defense Medical Institute, Taipei, Taiwan;* [5]*McKusick-Nathans Institute of Genetic Medicine, Howard Hughes Medical Institute, Johns Hopkins University School of Medicine, Baltimore, MD, USA*

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Abstract

Proline oxidase (POX), a mitochondrial inner-membrane protein, catalyzes the rate-limiting oxidation of proline to pyrroline-5-carboxylate (P5C). Previously we showed that overexpression of POX is associated with generation of reactive oxygen species (ROS) and apoptosis in POX-inducible colorectal cancer cells, DLD-1.POX. We also showed expression of mitochondrial MnSOD partially blunts POX-induced ROS generation and apoptosis. To further investigate the molecular basis of POX-induced apoptosis, we utilized the DLD-1.POX cells to show that cells overproducing POX exhibit an L-proline-dependent apoptotic response. The apoptotic effect is specific for L-proline, detectable at 0.2 mM, maximal at 1 mM, and occurs during 48–72 h following the addition of L-proline to cells with maximally induced POX. The apoptotic response is mitochondria-mediated with release of cytochrome *c*, activation of caspase-9, chromatin condensation/DNA fragmentation, and cell shrinkage. We conclude that in the presence of proline, high POX activity is sufficient to induce mitochondria-mediated apoptosis.

Key words: proline oxidase, apoptosis, reactive oxygen species, mitochondria, p53, proline-P5C cycle

Introduction

Proline oxidase (POX), also known as proline dehydrogenase (PRODH), is a mitochondrial inner membrane-associated protein catalyzing the rate-limiting oxidation/dehydrogenation of proline to pyrroline-5-carboxylate (P5C) using cytochrome *c* and FAD as electron and hydrogen acceptors [1–3]. The reaction is specific for proline; a paralogous

Address for offprints: James M. Phang, Metabolism and Cancer Susceptibility Section, Basic Research Laboratory, National Cancer Institute at Frederick, 1050 Boyles Street, Bldg 538, Room 115, NCI at Frederick, Frederick, MD, 20895, United States (E-mail: phang@ncifcrf.gov) (David Valle, BRB 519, 733 North Broadway, Johns Hopkins University School of Medicine, Baltimore, MD 21205, United States (E-mail: dvalle@jhmi.edu)

enzyme, hydroxyproline oxidase, catalyzes the oxidation of hydroxyproline [1]. In addition to its role in proline catabolism, the POX reaction, when coupled with P5C reductase, a cytosolic enzyme catalyzing reduced pyridine nucleotide-dependent conversion of P5C to proline, provides a mechanism to transfer redox potential between subcellular compartments [1, 4, 5]. In mammals, POX activity is developmentally regulated and tissue specific: highest in liver and kidney, lower in brain and heart, and undetectable in most other tissues [1]. The human POX structural gene (PRODH) maps to 22q11.2 and encodes a 600-amino acid protein with a predicted molecular weight of 68 kDa and an N-terminal mitochondrial targeting sequence of 25 amino acids [1, 6]. The structure of the orthologous protein in *E. coli* has been solved and the human enzyme modeled on this template [7]. The functional consequence of several common human mutations and their presumed structural basis has been determined [6].

Three independent studies using serial analysis of gene expression, subtractive hybridization and microarray technology have shown a > 5X induction in POX mRNA in apoptotic human colorectal cancer cells and bladder carcinoma cells following infection with a recombinant p53 adenovirus expressing p53 [8–11]. These observations suggest that P53-induced upregulation of POX is a general phenomenon in apoptosis. Using a POX-inducible colorectal cancer cell line DLD-1. POX, we recently showed that overexpression of POX induces L-proline-specific generation of reactive oxygen species (ROS) and apoptosis [5, 8]. Additionally, we found that overexpression of mitochondrial MnSOD, an antioxidant enzyme catalyzing the conversion of superoxide radical to hydrogen peroxide, partially blocks POX-induced apoptosis and on this basis concluded that ROS-initiated oxidative stress is the cause of POX-induced apoptosis [5]. Induction of apoptosis by increased generation of ROS is well documented [12–13]. Elevated expression of p53 leads to mitochondria-mediated apoptosis, in part, through induction of genes whose protein products are directly involved in the generation of ROS [14–16]. For example, several members of the Bcl-2 family that are regulated in p53-induced apoptosis are involved in ROS-mediated apoptosis [17–20]. BAX, a pro-apoptotic protein and a p53 downstream target, has been shown to be involved in the induction of a pro-oxidant state and ROS generation in neurons [19, 20]. In addition, we recently showed that PUMA [P53 Upregulated Modulator of Apoptosis], a direct p53 downstream target, induces apoptosis also in part through the BAX-dependent generation of superoxide radicals [21].

In addition to its role in ROS generation, POX is also a downstream effector in p53-mediated activation of the calcium/calmodulin-dependent phosphatase, calcineurin that regulates the nuclear factor of activated T cells (NFAT) to produce apoptosis in a variety of cancer cells [22]. However, the molecular mechanisms of POX-induced apoptosis are still largely unknown. Since POX is a mitochondrial inner membrane-associated protein capable of generation of ROS and functionally associated with oxidative phosphorylation, we hypothesized that POX-induced cell death is mediated by the mitochondrial pathway. Here we utilize the DLD-1.POX cell system and a variety of cellular and molecular assays to explore the POX-mediated apoptotic pathway.

Materials and methods

Cell lines and culture conditions

We cultured DLD-1.POX and DLD-1.vector cells, two ''Tet-Off'' inducible cell lines in non-induction (NonInd), proline-free, low glucose DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 250-μg/ml hygromycin B, 0.4-mg/ml G418 and 20-ng/ml doxycycline (DOX) as previously described [8, 21, 23]. For the induction of POX and cell death, we plated 7.0×10^5 DLD-POX cells in T25 flasks (Corning, Corning, NY) under NonInd conditions and the next day changed to induction medium (Ind, ''Tet-Off'') supplemented with various concentrations of L-proline. We harvested the cells over the next 6 days, performed cell counts using a hemacytometer and monitored cell death as previously described [21, 23].

POX assay

We assayed POX activity using a specific radioisotopic method [24]. Briefly, cells were harvested by trypsinization, washed in PBS, resuspended in 0.1-M KPO_4 (pH 7.2) and disrupted by sonication. The reaction mixture contained 0.1-M KPO_4 (pH 7.2), 10-μg/ml cytochrome *c*, 1-mM *ortho*-aminobenzaldehyde (OAB), 0.1-mM L-proline, 1-μCi ^{14}C L-proline, and 20 μg of cellular protein in a total volume of 250 μl. Following incubation at 37 °C for 30 min, the reactions were terminated by adding 50 μl of 2.5-mM OAB in 6 N HCl. We separated the dihydroquinazolinium compound, P5C-OAB, from precursor proline by ion exchange chromatography on 1-ml Dowex-50 resin [AG 50 W-X8, 100–200 mesh, hydrogen form, Bio-Rad] as previously described [24].

POX antiserum

We utilized rabbit polyclonal antibodies raised against the C-terminal 15 amino-acid peptide of human POX as previously described [6].

Detection of chromatin condensation and DNA fragmentation

We used Hoechst 33258 nuclear staining to detect chromatin condensation as previously described [23]. Multi-nucleated cells observed under the fluorescence microscope (Olympus, Tokyo, Japan) were considered to be apoptotic cells. To detect DNA fragmentation in total genomic DNA cells were cultured to 40% confluence in NonInd medium, rinsed in PBS and switched to Ind medium in the presence of various concentrations of proline and collected over a 7 day period. We collected approximately 5×10^5 cells and resuspended them in 20-μl lysis buffer (2-mM EDTA, 100 mM Tris pH 8.0, 0.8% SDS) with the addition of 2-μl RNase A and incubated at 37 °C for 30 min followed by addition of proteinase K (20 mg/ml) and incubation at 50 °C for 1.5 hr. DNA gel electrophoresis was conducted using 1.8% Seakem agaraose.

Flow cytometry analysis of apoptotic DLD-1.POX cells by propidium iodide (PI)

We assessed fragmentation of genomic DNA to sub-G1 DNA with the aid of fluorescence-activated cell sorter (FACS; Becton Dickinson, SanJose, CA) analysis as described [23]. Cells were grown for 4 days in the indicated medium, after which, cells were trypsinized, pelleted, and rinsed with PBS before fixing in 70% EtOH and 50-mM glycine, pH 2.0. Fixed cells were resuspended in propidium iodide (PI) buffer containing PBS, 0.1% glucose, 20-μg/ml PI, 100-Kunitz units/ml RNAase A, and incubated 1 hr at 37 °C before FACS analysis.

Immunofluorescence microscopy

DLD-1.POX cells were grown on glass coverslips, treated as indicated, fixed in 4% (v/v) paraformadehyde solution, blocked with 3% (w/v) bovine serum albumin (BSA), and then incubated in 3% BSA containing anti-cytochrome *c* antibody (1:1000, # 65981A, Pharmingen, San Diego, CA). Subsequently, coverslips were incubated in 3% BSA containing Texas Red-labeled secondary antibody (1:1000, # 715-075-151; Jackson ImmunoResearch, West Grove, PA), rinsed with HBS, mounted and subjected to fluorescence microscope analysis.

Subcellular fractionation and immunoblotting analysis

Subcellular fractionation of cytoplasmic and membrane fractions and immunoblotting assay were conducted as previously described [23]. In brief, cells were disrupted by polytron in cold sucrose buffer (0.25) M sucrose, 3.5-mM Tris and 1-mM EDTA, pH 7.4. Samples were then centrifuged at $100{,}000 \times g$, supernatants saved as cytoplasmic fractions and pellets saved as membrane fractions. Protein concentration was determined by the Bradford method (Bio-Rad, Hercules, CA). For immunoblotting, proteins were separated using 12% SDS-PAGE (25-μg protein/lane) and transferred to nylon membranes, which were then incubated with rabbit polyclonal anti-POX antiserum (1:500), anti-cytochrome *c* (7H8.2C12; Pharmingen), or anti-activated caspase-9 antibody (1:2,000; Pharmingen). Subsequently the membranes were hybridized with goat anti-rabbit-HRP secondary antibody (1:5,000) (Bio-Rad) and images were taken as previously described [23].

Results

Time-dependent induction of POX and proline-dependent apoptosis

To determine the extent and time course of POX induction in the DLD-1.POX cells, we harvested cells at daily intervals following transfer to induction (Ind) medium. POX specific activity increased more than ten-fold over 6 days from 4.5 nmol/hr/mg in the NonInd medium to 50 nmol/hr/mg in the Ind medium (Fig. 1A). POX protein, detected by immunoblotting, increased to a similar extent (Fig. 1B). The peak POX activity achieved in these cultured cells is similar to that in rat liver and about threefold higher than rat kidney cells [24]**.** POX activity (2 nmol product/hr/mg) was also detectable in the DLD-1.vector cells indicating that DLD-1 cells have some endogenous POX expression, even though it was not detectible on the Western. The difference between the DLD-1.vector and DLD-1.POX cells cultured in NonInd medium likely reflects leaky low-level expression of the pBI-ePOX construct. Parenthetically, the POX activity conferred by expression of the 1800 bp ORF POX cDNA and the size of the POX protein (~68 kDa) on immunoblots strongly support our prediction of the size of full length 600-aa POX protein [6]. To determine the growth of DLD-1.POX cells under induction in the presence or absence of proline or other amino acids, we used MEM medium plus 10% dialyzed serum and proline-free nonessential amino acids to rid any exogenous sources of proline in the cell growth and death assays. To investigate whether or not POX-induced cell death is proline-dependent, we harvested and counted attached DLD-1.POX cells grown in Ind medium for 4 days supplemented with variable amounts of proline (Fig. 2A). We found that DLD-1.POX cells cultured in Ind medium in the absence of proline had lower growth rates than DLD-1.POX cells cultured in NonInd medium with zero proline (Fig. 2A, bars 2 and 3). Presumably this was due to the

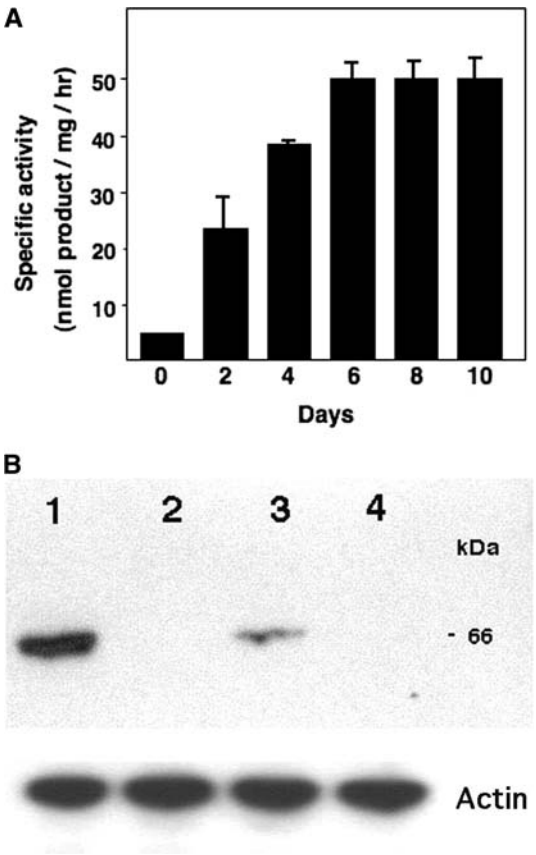

Fig. 1. (A) Time course of induction of POX activity in DLD-1.POX cells. DLD-1.POX cells were transferred to the induction medium at 0 days. Cells were harvested at the indicated time for assay of POX activity. (B) Immunoblot analysis of POX induction. Cells were cultured in the indicated medium for 4 days, harvested and sonicated. Each lane contains 20 μg of cell protein. Lane 1, DLD-1.POX cells cultured in Ind medium; lane 2, DLD-1.vector cells cultured in Ind medium; lane 3, DLD-1.POX cells cultured in NonInd medium; lane 4, DLD-1.vector cells cultured in NonInd medium. The same samples were run on a parallel blot for β-actin.

accessibility of endogenous proline. Nevertheless, cells cultured in Ind medium in the presence of 0.2-mM proline (Fig. 2A, bar 4), 1-mM proline (Fig. 2A, bar 5), or 5-mM proline (Fig. 2A, bar 6) had increased cell death corresponding to the increased proline concentration. The lower growth rate in DLD-1.POX in NonInd medium with 5.0-mM proline was lower than DLD-1.vector consistent with the finding of leaky expression (Fig. 2A, bars 7 and 8). These results were consistent with those of our previous observations in DLD-1 cells that induction of POX in the presence of 1 or 5 mM proline has a profound effect on cell proliferation (Fig. 2B) [5, 8]. We also found that other amino acids (e.g., L-glutamate, L-glutamine, or L-ornithine) had no effect on the growth of induced DLD-1.POX cells (data not shown).

To determine if the increased cell death was caused by the induction of apoptosis, we showed that induced DLD-1.POX cells in the presence of 5-mM proline exhibited time-dependent DNA fragmentation (laddering) by 4 days after induction (Fig. 2C), suggesting that these cells are undergoing POX-induced and proline-dependent apoptosis. We also performed the analysis of DNA content, cell cycle progression and apoptosis in induced DLD-1.POX cells using PI staining in combination with flow cytometry as described [21, 23]. We found a > tenfold increase of the sub-G1 population in POX-induced cells grown in 5-mM proline indicating increase of apoptosis (Fig. 3D & E). We also found that induction of apoptosis in DLD-1.POX cells is proline-dose-dependent. The effect is apparent at 0.5-mM and maximal at 1.0 mM (Fig. 3E). The observed increase in S phase and G1 observed in Fig. 3D is consistent with a block in S phase, with cells in G2 cycling to phase sub-G1. In contrast, DLD-1.POX cells cultured in NonInd medium or NonInd plus 5 mM proline medium grow not significantly different from control (Fig. 3B, C & E).

Cytochrome c release and activation of caspase-9 in apoptotic DLD-1.POX cells

Release of cytochrome *c* from inter-mitochondrial space to cytosol and subsequent activation of caspase-9 are the characteristics of mitochondria-mediated apoptosis. We first used the anti-cytochrome *c* antibody to conduct immunofluorescence assay to localize cytochrome *c* in the POX-induced cells in the presence and absence of proline. As shown in Fig. 4 panels a & b, cytochrome *c* is located in the mitochondria when DLD-1.POX cells were cultured in NonInd medium. In contrast, after overexpressing POX in DLD-1 cells in the presence of proline for 4 days, cytochrome *c* displayed cytosolic localization, indicating cytochrome *c* was released from mitochondria (Fig. 4, panel b). To verify this result we conducted immunoblotting analysis of the cytosolic fractions of induced DLD-1.POX cells in the presence of proline at various time intervals and showed that there was a time-dependent induction of cytochrome *c* release starting from day 2 in apoptotic DLD-1.POX cells (Fig. 5). We also examined activation of caspase-9 by immunoblotting analysis and showed that POX overexpression in the presence of proline also induces time-dependent activation of caspase-9 (Fig. 5).

Discussion

In p53-null DLD-1 colorectal cancer cells expressing high POX activity, we show that the addition of L-proline (0.5 mM or greater) is sufficient to induce mitochondria-mediated apoptosis characterized by release of cytochrome *c*,

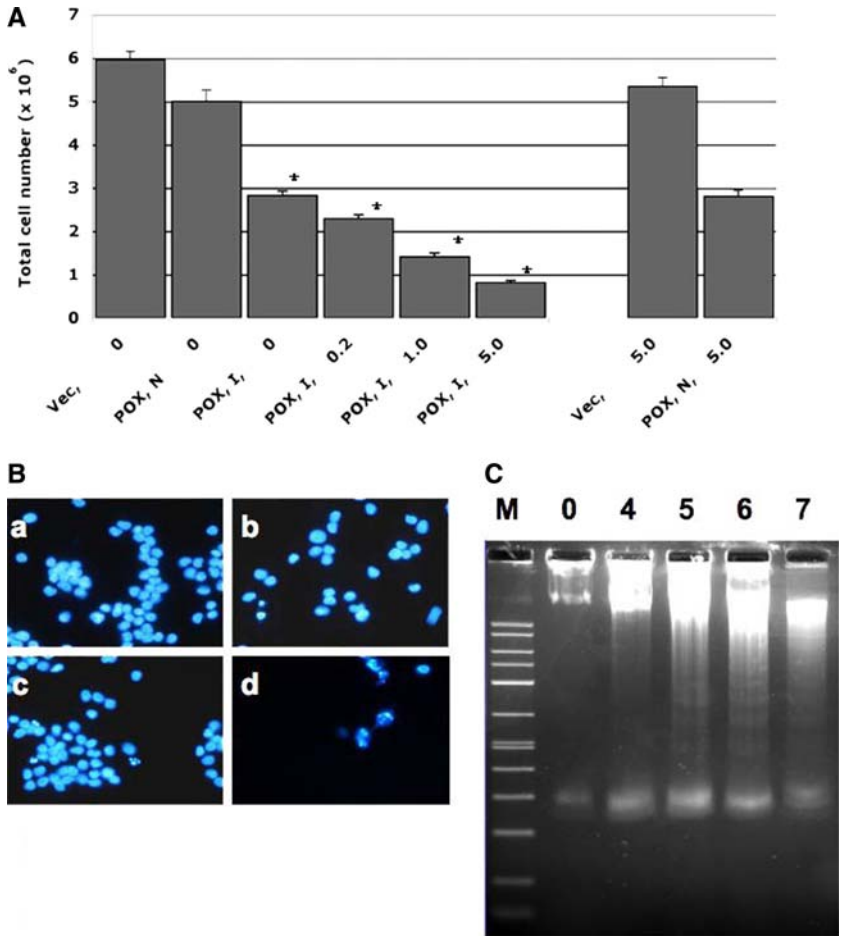

Fig. 2. Overexpression of POX in the presence of proline induces apoptosis. (A) POX-induced cell death is proline-dependent. Attached DLD-1.POX cells grown in the indicated medium for 4 days were harvested for cell count. Vec, 0, DLD-1.vector cells in the absence of proline; POX, N, 0, DLD-1.POX cells, NonInd medium in the absence of proline; POX, I, 0, DLD-1.POX cells, Ind medium in the absence of proline; POX, I, 0.2, DLD-1.POX cells, Ind medium with 0.2-mM proline; POX, I, 1.0, DLD-1.POX cells, Ind medium with 1.0-mM proline, POX, I, 5.0, DLD-1.POX cells, Ind medium with 5.0mM proline; Vec, 5.0, DLD-1.vector cells in 5.0-mM proline; POX, N, 5.0, DLD-1.POX cells in NonInd medium with 5.0-mM proline. All data represent mean + SEM of at least 3 determinations. *represents statistical significance ($P < 0.01$) compared to DLD-1.POX, NonInd medium with 0 proline (bar 2). (B) Hoechst staining of cells cultured in the indicated media for 4 days. (a) DLD-1.vector cells cultured in Ind-0; (b) DLD-1.vector cells cultured in Ind-5; (c) DLD-1.POX cells cultured in Ind-0; (d) DLD-1.POX cells cultured in Ind-5. The oval blue structures are nuclei. Irregular nuclei with pale blebs are the nuclei of apoptotic cells undergoing chromatin condensation and DNA fragmentation. Note in (d) the cell number is greatly reduced with nearly all the remaining cells undergoing apoptosis as indicated by the nuclear blebs. (C) Detection of DNA fragmentation by gel electrophoresis. Total genomic DNA was isolated from DLD-1.POX cells cultured in Ind-5 for the number of days indicated at the top of each lane. The DNA marker is a 1-kb ladder.

activation of caspase 9, nuclear condensation/fragmentation and end-stage cell shrinkage. This occurs apparently independent of p53. Neither POX induction nor high extracellular proline were, by themselves, sufficient for this apoptotic response. The requirement for proline were specific and the response occurred during 48–72 hr following proline supplementation. Previously, we showed an increase of cellular ROS following addition of proline in induced DLD-1.POX cells [5]. Moreover, agents that reduced ROS levels (e.g. NAC and MnSOD) blunted the apoptotic response [5]. Taken together, these observations strongly suggest that in the presence of extracellular proline concentrations about twice the normal upper limit of plasma proline levels (normal range 51–271 μM) POX-catalyzed proline oxidation reaction generates ROS in excess of the cells antioxidation capacity and stimulates mitochondria-mediated apoptosis (Fig. 6). In addition, overexpression of POX in the presence of proline is expected to produce increased amounts of its immediate product, P5C, possibly perturbing other signaling molecules that involve in cell death regulation [25, 26] (Fig. 6).

We previously showed that it is the generation of superoxide radicals not hydrogen peroxide that cause apoptosis in the induced DLD-1.POX cells exposed to high proline [5].

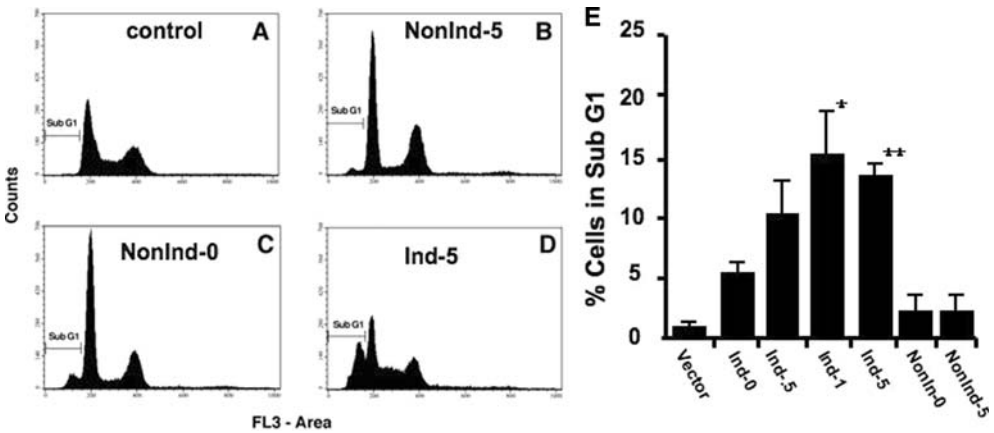

Fig. 3. Flow cytometry analysis on (A) DLD-1.vector cells cultured in Ind-0; (B) DLD-1.POX cells cultured in NonInd-5; (C) DLD-1.POX in NonInd-0; (D) DLD-1.POX in Ind-5. (E) Histogram of percent of sub-G1 population in a proline-dose-dependent apoptosis assay by flow cytometry assay on DLD-1.vector or DLD-1.POX cells in the presence of various proline concentrations. Data represent mean + SE of at least 3 determinations. Compared to DLD-1.POX in NonInd medium, the differences were statistically significant ($*p < 0.05$; $**p < 0.01$).

Although the leakage of electrons from the electron transport chain is a plausible mechanism, an alternative mechanism has been suggested. Giorgio and colleagues recently showed that $p66^{shc}$, a genetic determinant of life span in mammals predominantly localized within the mitochondrial intermembrane space, functions as a redox enzyme that utilizes reducing equivalents of the mitochondrial electron transport chain to generate ROS (predominantly H_2O_2) within mitochondria through the oxidation of cytochrome *c*. Mutant $p66^{shc}$ defective in redox activity failed to induce mitochondrial ROS generation and therefore unable to induce apoptosis in cells [27]. These results suggest that ROS are not just accidental and inevitable by-products of aerobic respiratory chain; rather, they can be specifically generated and utilized as signaling molecules in apoptosis by specialized enzymes such as $p66^{shc}$ or, perhaps, POX. Furthermore, although POX appears to donate electrons to cytochrome *c* [1, 2], the physical relationship between these two proteins is uncertain and this may be of importance. Furthermore, P5C or its tautomer, glutamic-γ-semialdehyde, provide another possibility. The latter has a carbonyl moiety, which is a reactive nucleophile. It has been proposed by others that apoptosis can be induced by carbonyl species [28]. In this regard, glutamic-γ-semialdehyde or P5C has been previously described as "toxic." [4, 5] Finally, P5C, is an intermediate in the metabolic interconversions of proline, ornithine, and glutamate that freely traverses mitochondrial membranes. Thus, the POX reaction, together with that catalyzed by P5CR, comprise a proline/P5C cycle that perturbs the cytosolic NAD[P]H/$NAD[P]^+$ ratio [4, 5]. Perhaps excessive flux in the POX catalyzed reaction disturbs the cellular redox state, P5C

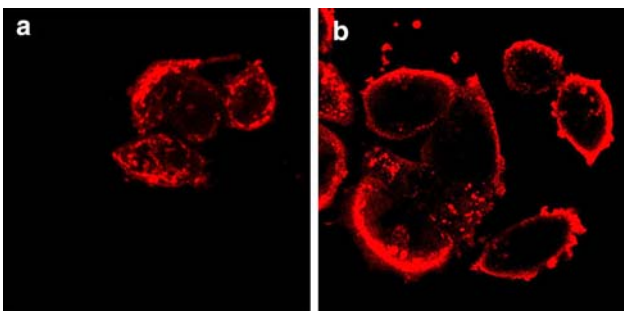

Fig. 4. Immunolocalization of cytochrome *c* in DLD-1.POX cells grown in the indicated media for 4 days. (a) NonInd-0; (b) Ind-5. Cytochrome *c* is in red.

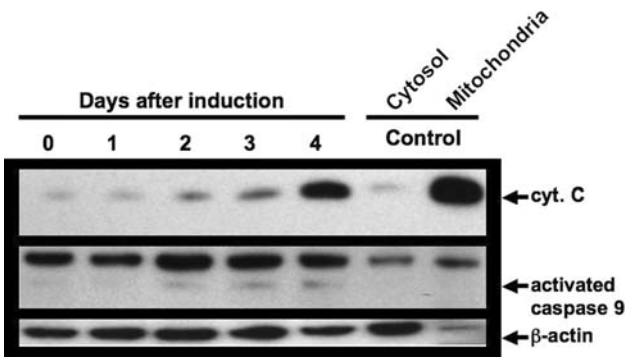

Fig. 5. Immunoblot analysis of cytochrome *c* and caspase-9. Cytosolic extracts were prepared from DLD-1.POX cells cultured in Ind medium for the indicated times. Cytosolic and mitochondrial fractions isolated from DLD-1 .POX cells grown in NonInd medium were used as control. Release of cytochrome *c* and activation of caspase-9 were observed 2 days after induction in induced DLD-1.POX cells. β-actin staining of the same lanes was used to provide a loading control.

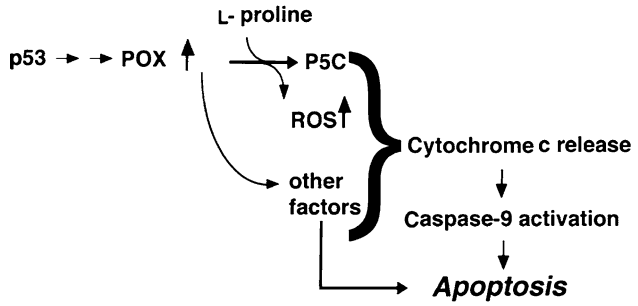

Fig. 6. Hypothetical model of POX/proline-induced ROS generation and apoptosis. Cells expressing high POX activity in the presence of proline overproduce P5C, ROS, or possibly some other factors with resultant apoptosis mediated by the mitochondrial pathway.

homeostasis, mitochondrial FAD/FADH2 and/or cytosolic NAD[P]H/NAD[P]$^+$ ratios to an extent that contributes to the apoptotic response [29, 30].

Regardless of the mechanism, our observations raise the question of a possible role of proline/POX-mediated apoptosis in vivo. The specific activity of POX in the induced DLD-1 cells is similar to that in rat liver and within an order of magnitude of that in other rat tissues [31, 32]. Furthermore, the concentration of proline required to induce apoptosis in the induced DLD-1 POX cells is only about two fold higher than that normally present in the plasma of fasting individuals (51–271 μM). Thus, it is possible that proline-induced apoptosis in cells with high POX activity may have some physiologic role in specific cells or tissues under certain conditions. For example, POX/proline stimulus for apoptosis may play an in vivo role in certain cells or tissues, such as thymus, brain, pre- or neonatal intestinal epithelial cells, in spaciotemporally regulated manner. Interestingly, these processes would be expected to be affected by inborn errors of proline metabolism. In humans, POX deficiency causes Type I Hyperprolinemia (HPI), a rare and poorly characterized inborn error [1, 6]. A spontaneously appearing, POX deficient mouse strain, PRO/Re, with abnormal sensory motor gating also has been described [33, 34] and shown to be homozygous for a nonsense mutation, E566X [35]. If proline-induced apoptosis in cells expressing high POX activity has a normal physiologic function, this pro-apoptotic mechanism would be blocked by POX deficiency and could contribute to some or all of the phenotypic features of HPI. The PRO/Re mice provide a model for investigating possible involvement of POX in apoptosis. A second human inborn error associated with elevation of plasma proline, Type II Hyperprolinemia (HPII), is caused by deficiency of P5C dehydrogenase [1, 36]. If the proline/POX-mediated apoptosis has some *in vivo* role, these individuals with whose plasma proline concentrations range from 500 to 2000 μM might have excessive apoptosis in those tissues with adequate POX levels. Alternatively, the role of proline/POX in apoptosis may be limited to conditions of nutritional or bioenergetic stress, i.e., as an alternative source of metabolic energy for apoptosis in lieu of NADH in mitochondria. Even ROS are derived from electrons from NADH or in some circumstances from succinate. Under conditions of nutritional stress when these sources are unavailable, the proline/POX system may provide an alternative source.

Finally, it will be important to investigate possible cross-talk between POX, cytochrome *c*, FAD, protein components of the electron transport chain and mitochondria-independent apoptotic pathways, and to explore the feasibility of using POX in cancer prevention, diagnosis, prognosis, and treatment.

Acknowledgments

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References

1. Phang JM, Hu CA, Valle D: Disorders of proline and hydroxproline metabolism. In: CR Scriver, AL Beaudet, WS Sly and D Valle (eds), Metabolic and Molecular Bases of Inherited Disease. McGraw Hill Press: New York, 2001, pp. 1821–1838
2. Johnson AB, Strecker JH: The interconversion of glutamic acid and proline. IV. The oxidation of proline by rat liver mitochondria. J Biol Chem 237: 1876–1882, 1962
3. Adams E, Frank L: Metabolism of proline and the hydroxyprolines. Annu Rev Biochem 49: 1005–1061, 1980
4. Phang JM: The regulatory functions of proline and pyrroline-5-carboxylic acid. Curr Top Cell Regul 25: 91–132, 1985
5. Liu Y, Borchert GL, Donald SP, Surazynski A, Hu CA, Weydert CJ, Oberley LW, Phang JM: MnSOD inhibits proline oxidase-induced apoptosis in colorectal cancer cells. Carcinogenesis 26: 1335–1342, 2005
6. Bender HU, Almashanu S, Steel G, Hu CA A, Lin WW, Willis A, Pulver A, Valle D: Functional consequences of *PRODH* missense mutations. Am J Hum Genet 76: 409–420, 2005
7. Lee YH, Nadaraia S, Gu D, Becker DF, Tanner JJ: Structure of the proline dehydrogenase domain of the multifunctional PutA flavoprotein. Nat Struct Biol 10: 109–114, 2003
8. Donald SP, Sun XY, Hu CA, Yu J, Mei JM, Valle D, Phang JM: Proline oxidase, encoded by p53-induced gene-6, catalyzes the generation of proline-dependent reactive oxygen species. Cancer Res 61: 1810–1815, 2001
9. Maxwell SA, Davis GE: Differential gene expression in p53-mediated apoptosis-resistant vs. apoptosis-sensitive tumor cell lines. Proc Natl Acad Sci U S A 97: 13009–13014, 2000
10. Maxwell SA, Rivera A: Proline oxidase induces apoptosis in tumor cells, and its expression is frequently absent or reduced in renal carcinomas. J Biol Chem 278: 9784–9789, 2003

11. Zhu J, Jiang J, Zhou W, Zhu K, Chen X: Differential regulation of cellular target genes by p53 devoid of the PXXP motifs with impaired apoptotic activity. Oncogene 18: 2149–2155, 1999
12. Raha S, Robinson RH: Miotchondria, oxygen free radicals, and apoptosis. Am J Med Genet 106: 62–70, 2001
13. Martindale JL, Holbrook NJ: Cellular response to oxidative stress: signaling for suicide and survival. J Cell Physiol 192: 1–15, 2002
14. Polyak K, Xia Y, Zweier JL, Kinzler KW, Vogelstein B: A model for p53-induced apoptosis. Nature 389: 300–305, 1997
15. Johnson TM, Yu ZX, Ferrans VJ, Lowenstein RA, Finkel T: Reactive oxygen species are downstream mediators of p53-dependent apoptosis. Proc Natl Acad Sci USA 93: 1848–1852, 1996
16. Vogelstein B, Lane D, Levine AJ: Surfing the p53 network. Nature 408: 307–310, 2000
17. Miyashita T, Krajewski S, Krajewska M, Wang HG, Lin HK, Lieberman DA, Hoffman B, Reed JC: Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression *in vitro* and *in vivo*. Oncogene 9: 1799–1805, 1994
18. Miyashita T, Reed JC: Tumor suppressor p53 is a activator of the human bax gene. Cell 80: 293–299, 1995
19. Kirkland RA, Franklin JL: Bax, reactive oxygen, and cytochrome *c* release in neuronal apoptosis. Antioxid Redox Signal 5: 589–596, 2003
20. Kirkland RA, Windelborn JA, Kasprzak JM, Franklin JL: A Bax-induced pro-oxidant state is critical for cytochrome *c* release during programmed neuronal death. J Neurosci 22: 6480–6490, 2002
21. Liu Z, Lu H, Shi H, Du Y, Yu Gu J. S, Chen X, Liu KJ, Hu CAA: PUMA overexpression induces ROS generation and proteasome-mediated stathmin degradation in colorectal cancer cells. Cancer Res 65: 1647–1654, 2005
22. Rivera A, Maxwell SA: The p53-induced gene-6 (proline oxidase) mediates apoptosis through a calcineurin-dependent pathway. J Biol Chem 280: 29346–29354, 2005
23. Liu Z, Lu H, Jiang Z, Pastuszyn A, Hu CAA: Apolipoprotein L6, a novel pro-apoptotic BH3-only protein, induces mitochondria-mediated apoptosis in cancer cells. Mol Can Res 3: 21–31, 2005
24. Phang JM, Downing SJ, Valle DL, Kowaloff EM: A radioisotopic assay for proline oxidase activity. J Lab Clin Med 85: 312–317, 1975
25. Ferri KF, Kroemer G: Organelle-specific initiation of cell death pathways. Nat Cell Biol 3: E255–E263, 2001
26. Liu Y, Borchert GL, Syrazynski A, Hu C-A, Phang JM 2006 Proline oxidase activates both intrinsic and extrinsic pathways for apoptosis: the role of ROS/superoxides, NFAT and MEK/ERK signaling. Oncogene EPub April 17
27. Giorgio M, Migliaccio E, Orsini F, Paolucci D, Moroni M, Contursi C, Pelliccia G, Luzi L, Minucci S, Marcaccio M, Pinton P, Rizzuto R, Bernardi P, Paolucci F, Pelicci PG: Electron transfer between cytochrome *c* and p66Shc generates reactive oxygen species that trigger mitochondrial apoptosis. Cell 29: 221–233, 2005
28. Akhand AA, Du J, Liu W, Hossain K, Miyata T, Nagase F, Kato M, Suzuki H, Nakashima I: Redox-linked cell surface-oriented signaling for T-cell death. Antioxid Redox Signal 4: 445–454, 2002
29. Butow RA, Avadhani NG: Mitochondrial signaling: The retrograde response. Mol Cell 14: 1–15, 2004
30. Skulachev VP: Bioenergetic aspects of apoptosis, necrosis and mitoptosis. Apoptosis 11: 473–485, 2006
31. Kowaloff EM, Granger AS, Phang JM: Alterations in proline metabolic enzymes with mammalian development. Metabolism 25: 1087–1094, 1976
32. Downing SJ, Phang JM, Kowaloff EM, Valle D, Smith RJ: Proline oxidase in cultured mammalian cells. J Cell Physiol 91: 369–376, 1977
33. Blake RL: Animal model for hyperprolinaemia: deficiency of mouse proline oxidase activity. Biochem J 129: 987–989, 1972
34. Blake RL, Hall JG, Russell ES: Mitochondrial proline dehydrogenase deficiency in hyperprolinemic Biochem. Genet 14: 739–757, 1976
35. Gogos JA, Santha M, Takacs Z, Beck KD, Luine V, Lucas LR, Nadler JV, Karayiorgou M: The gene encoding proline dehydrogenase modulates sensorimotor gating in mice. Nat Genet 21: 434–439, 1999
36. Geraghty MT, Vaughn D, Nicholson AJ, Lin WW, Jimenez-Sanchez G, Obie C, Flynn MP, Valle D, Hu CA: Mutations in the Delta1-pyrroline 5-carboxylate dehydrogenase gene cause type II hyperprolinemia. Hum Mol Genet 7: 1411–1415, 1998