

# Proline Metabolism and Microenvironmental Stress

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## Key Words

cancer, collagen, bioenergetics, tumor suppressor, microRNA, oxLDL

## Abstract

Proline, the only proteinogenic secondary amino acid, is metabolized by its own family of enzymes responding to metabolic stress and participating in metabolic signaling. Collagen in extracellular matrix, connective tissue, and bone is an abundant reservoir for proline. Matrix metalloproteinases degrading collagen are activated during stress to make proline available, and proline oxidase, the first enzyme in proline degradation, is induced by p53, peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) and its ligands, and by AMP-activated protein kinase downregulating mTOR. Metabolism of proline generates electrons to produce ROS and initiates a variety of downstream effects, including blockade of the cell cycle, autophagy, and apoptosis. The electrons can also enter the electron transport chain to produce adenosine triphosphate for survival under nutrient stress. Pyrroline-5-carboxylate, the product of proline oxidation, is recycled back to proline with redox transfers or is sequentially converted to glutamate and alpha-ketoglutarate. The latter augments the prolyl hydroxylation of hypoxia-inducible factor-1 $\alpha$  and its proteasomal degradation. These effects of proline oxidase, as well as its decreased levels in tumors, support its role as a tumor suppressor. The mechanism for its decrease is mediated by a specific microRNA. The metabolic signaling by proline oxidase between oxidized low-density lipoproteins and autophagy provides a functional link between obesity and increased cancer risk.

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## INTRODUCTION

### Renewed Focus on Metabolism in Cancer

The recent upsurge of interest in cancer metabolism was due, at least in part, to the realization that cancer genomics can be best understood on the basis of metabolic pathways (19, 25); mutations in signaling pathways often result in alterations in common metabolic endpoints. The frequently mentioned findings of Otto Warburg, that tumor tissues have shifted from oxidative phosphorylation to aerobic glycolysis, provide the archetype of such a common denominator (109). Some of these mechanisms remain speculative, but robust evidence supporting a number of models has emerged (18, 105). Therapeutic targeting of these

converging metabolic pathways has distinct advantages. Thus, identification of these pathways has implications that are more than academic.

Many regulatory concepts in cancer metabolism have derived from the homeostasis paradigm. Historically, research in metabolism has been coupled with endocrinology, and the latter, with the exception of reproductive systems, centers on homeostasis and bioenergetics. It is not surprising that even in the approach to understanding the Warburg hypothesis, the evoked metabolic mechanisms are based on a bioenergetic model. A contrasting model invokes acute and transitory responses and adaptations in the microenvironment, e.g., during wound healing and also during tumor invasion (81, 102). These models include inflammation, changes in cell-matrix interactions, e.g., detachment from the basement membrane, activation of matrix metalloproteinases (MMPs), and the epithelial-mesenchymal transition. This review describes mechanisms offered by the metabolism of proline that bridge the microenvironmental model and the homeostatic model for metabolism.

In considering the microenvironment, metabolism may play a critical role in three stress responses: (a) With genotoxic stress, damage to the genome initiates halting of the cell cycle and engagement of repair processes. If these repairs are unsuccessful, an apoptotic cascade ensues. Although the molecular mechanisms switching from repair to programmed cell death are not fully understood, the metabolic adaptation is critical. (b) With inflammatory stress, as occurs with wound healing and exposure to pathologic microorganisms, the metabolic requirements must satisfy special needs. (c) A central example of metabolic adaptation in the microenvironment relates to tumor progression and metastasis (15, 79). Here, the metabolic needs of the tumor are arrayed against those of host defense mechanisms. After loss of basement membrane attachment and blood supply, tumor cells must run the gauntlet of metabolic stress to survive and to proliferate (19, 88, 105). Various cellular elements in its microenvironment will

attack the tumor cell and must mobilize various metabolic processes to do so. These underlying concepts are helpful in our understanding of the metabolic needs in cancer (88, 105).

## Nutrition

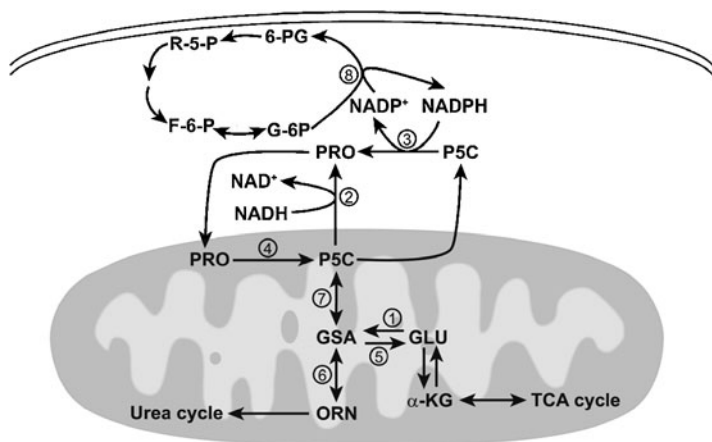
Although recent reviewers have considered state-of-the-art cellular paradigms in evaluating the effects of nutrition, they have not emphasized new and changing therapeutic modalities. Traditional views consider nutrients taken orally, processed in the digestive tract, and delivered to local sites by the circulation. An equally important consideration, at least for so-called nonessential nutrients, is the endogenous pathways for biosynthesis. In nutrition, amino acids have been dichotomized into essential and nonessential (87). However, for stress responses to the microenvironment, nonessentialness based on redundant pathways responsive to control is critical. Of course, the storage and mobilization of both glucose and lipids have been thoroughly investigated and described in the context of energetics. The storage of glucose as glycogen and its mobilization in a hormone-regulated process have been thoroughly studied. Similarly, both lipogenesis and lipolysis and their regulation by various hormones have been areas of elegant research. Although recently there has been considerable emphasis on proteolysis, especially ubiquitinylation-mediated, proteasomal degradation (116), the interpretation of such proteolysis has emphasized the control of specific proteins. Little attention has been given to proteins as a reservoir of amino acids, for proteinogenesis, as substrate for energetics, or as mediators of cellular signaling. These considerations offer new perspectives on nutritional metabolism.

The delivery of specialized nutrient formulations has revolutionized parenteral nutrition, especially in the postsurgical environment (110). The first step is to supply the entire spectrum of nutrients including amino acids, micronutrients, and lipids in addition to physiologic salts and glucose. Importantly, the discovery that glutamine added at supraphysio-

logic concentrations can accelerate postsurgical healing, transcends nutrient replacement (96). Thus, nutrients can be used pharmacologically, at least in some situations. Although the skillful placement of catheters has been used to deliver a variety of pharmacologic agents, e.g., clotting agents in an area of developing clots or to dissolve forming intravascular clots (45, 106), the delivery of specific nutrients has not been used. In the future, especially as nutrients are shown to have specific, localized effects, the specific delivery into an evolving pathologic site may be developed as an adjunctive therapy. Finally, the recent development of nanotechnology may open up new vistas in targeted delivery, not only of pharmacologic agents but also of micro- as well as macronutrients (44, 97). As mentioned above, the dividing line between nutritional and pharmacologic agents may be artificial and arbitrary, and nutritional agents, if delivered at the right organ or tissue site and at the appropriate concentrations, may have pharmacologic effects.

## Proline Metabolism

Because its  $\alpha$ -amino nitrogen is contained within a pyrrolidine ring, proline is the only proteinogenic secondary amino acid, and it has special functions in biology (87, 92). Its unique role in proteins is well-recognized—providing not only physical stability to proteins, e.g., collagen, but also serving as the basis for molecular recognition and signaling (66). In fact, it has been suggested that all protein-protein interaction is based on proline-dependent recognition. In a similar fashion, the metabolism of proline is distinct from that of primary amino acids (1, 2, 87). What is not usually appreciated is that collagen is the most abundant protein in the body, and 25% of the residues in collagen are proline together with hydroxyproline (22). The special structure of proline precludes its being substrate for the generic enzymes metabolizing most amino acids. Instead, a special family of enzymes has evolved with its own tissue distribution, subcellular localization, and mechanisms of regulation. The



**Figure 1**

Schematic of pathways for proline metabolism. The pentose phosphate pathway is simplified. Not all steps are shown. 6-PG, 6-phosphogluconate; F-6-P, fructose-6-phosphate; G-6P, glucose-6-phosphate; GLU, glutamic acid; GSA, glutamic semialdehyde; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; ORN, ornithine; P5C, pyrroline-5-carboxylate; PRO, proline; R-5-P, ribose-5-phosphate; TCA cycle, tricarboxylic acid cycle. Numerals designate the following enzymes: 1, P5C synthase; 2, P5C reductase 1; 3, P5C reductase 2; 4, proline oxidase (a.k.a. proline dehydrogenase); 5, P5C dehydrogenase; 6, ornithine aminotransferases; 7, spontaneous; 8, glucose-6-phosphate dehydrogenase.

physiologic/pathophysiologic importance of these unique aspects of proline metabolism has not been appreciated until the past decade. The reluctance of the scientific community to investigate proline has several reasons. First, proline is a nonessential amino acid, as has been previously mentioned, thus it has been relegated to lesser importance than essential amino acids. Another factor that has led to the neglect of proline metabolism is that its metabolism is not easily monitored in body fluids. Unlike glutamine and alanine, which are mediators for intertissue carbon and nitrogen exchange, the effects of proline metabolism are primarily microenvironmental. Even when proline is mobilized from abundant sources of protein, the effects of its metabolism may not be reflected by changes in the circulating body fluids.

For a description of the proline metabolic enzymes, the reader is referred to recent

reviews (38, 88, 91). Advances in the biochemistry, structure, and genomics of these enzymes have added much to our understanding. The discovery of clinical syndromes with mutations of these specific enzymes has contributed to this understanding (38, 95). Furthermore, the finding that specific enzymes are regulated by various response pathways has provided insight into their regulatory function. Unquestionably, recent studies have robustly supported the proposal that proline, pyrroline-5-carboxylic, and their metabolism have regulatory functions. A detailed description of the genetic, molecular, and biochemical properties of these enzymes is beyond the focus of this review. The reader is referred to a number of recent publications (38, 91, 95). However, in order to discuss the relevant metabolism in an understandable fashion, a brief description of these enzymes is necessary (Figure 1).

Proline is interconvertible with glutamate and arginine (1, 2, 87). Although the steps between proline and glutamate are direct, arginine must first be converted to ornithine; this conversion is an important step in the urea cycle. In terms of these interconversions,  $\Delta^1$ -pyrroline-5-carboxylic acid (P5C) or its straight-chain tautomer, glutamic- $\gamma$ -semialdehyde (GSA) is the central intermediate. The factors that determine whether this intermediate is in the ring or chain form in situ are not well understood (87), but at physiologic pH the chain form would be favored. The reactivity of the carbonyl group in the straight chain may necessitate special channeling or sequestration by chaperones. In this context, the discovery of the specialized structure of P5C reductase provides some understanding of the function of the proline metabolic system (87).

Although each member of the triad of proline, ornithine, and glutamate deserves focus, this proline-centric review organizes the metabolic pathway as catalyzed by proline-synthesizing enzymes and proline degradative enzymes. This classical dichotomy is based on the proteinogenic function of proline, but it has limited usefulness when considering the regulatory functions of the metabolic system

(87). In the context of regulation, a more useful dichotomy would be P5C-producing enzymes and P5C-utilizing enzymes.

**P5C-producing enzymes.** Ornithine- $\delta$ -aminotransferase (OAT) converts ornithine in a reversible reaction to GSA (1, 3). Although reversible, the reaction favors the production of the latter, but under certain situations, for example, in the neonate, there is a net conversion of proline to arginine mediated by the reverse reaction of OAT. Thus, OAT is the only enzyme in the system that is bi-directional. Although the amino donor/acceptor is usually considered to be  $\alpha$ -ketoglutarate/glutamate (aKG), pyruvate/alanine and glyoxylate/glycine can also mediate the reaction. The enzyme is localized to the mitochondrial matrix (104). P5C synthase catalyzes the two-step reaction from glutamate to GSA requiring first an adenosine triphosphate (ATP)-dependent phosphorylation, followed by a reduction step with reduced pyridine nucleotide [NAD(P)H] as cofactor. There are two known isozymes of P5CS, a long form and a short form (40, 41). The short form is located primarily in the intestine and is sensitive to feedback inhibition by ornithine; thus it seems to function primarily to convert glutamate to arginine. The long form is ubiquitously expressed and thereby interpreted as primarily a proline-producing isozyme. Proline oxidase, a.k.a. proline dehydrogenase (POX/PRODH), is a mitochondrial inner-membrane enzyme catalyzing the transfer of electrons from proline (111), producing P5C. The pair of electrons can be used either for direct reduction of oxygen to form superoxide or is transferred into the electron transport chain for oxidative phosphorylation (101, 111). The function of POX/PRODH in cancer is discussed below.

**P5C-utilizing enzymes.** P5C reductase catalyzes the conversion of P5C to proline (1). Two isozymes are known to exist, P5CR1 and P5CR2 (64), encoded by distinct genes P5C reductase 1 and 2 (*PYCR1* and *PYCR2*),

respectively (38). The former prefers NADH as cofactor and has a high-capacity activity, whereas the latter prefers NADPH and has lower capacity (64, 73). Both isozymes are widely distributed; even the erythrocyte from which P5CR2 has been purified probably has some residual P5CR1 activity. Although the enzymes are easily recovered from the cytosol, their association with either mitochondrial outer membranes or cytosolic surface of plasma membranes is likely (95). The enzyme structure has been characterized as a decamer with a structure similar to proteins with transporter or chaperone function (72). P5C dehydrogenase or GSA dehydrogenase converts GSA to glutamate with  $\text{NAD}^+$  as cofactor (91). The enzyme is related to the family of aldehyde dehydrogenases. Although early workers found the enzyme in both mitochondria and cytosol, it is likely that it is primarily located in the mitochondrial matrix (1).

### Inborn Errors of the Proline Metabolic Pathway

A detailed discussion of the human genetic disorders in the enzymes of the proline metabolic pathway is beyond the scope of this review. The reader is referred to a recent review by Hu et al. (38) as well as a more comprehensive discussion in the *Metabolic and Molecular Basis of Human Diseases* (91). Nevertheless, it should be mentioned here that human disorders with deficiencies of OAT (104) and P5C dehydrogenase (91) are well documented. Recently, mutations in P5CS (4, 5) and PYCR1 (95) have been described. Abnormal development has been associated with both defects. In the latter, decreased resistance to oxidizing insult is of special interest. In spite of intense investigation, mutations in POX/PRODH as the cause of increased risk of neuropsychiatric disorders, e.g., schizophrenia, is supported by a variety of evidence but remains a topic of debate (6, 112). Since these linkages have been determined by epidemiological genetics, subtle differences in the study populations may be critical. Additionally, a confounding factor may

be that HYPOX/PRODH2 can provide compensatory regulatory functions (13) in patients with defects in POX/PRODH. Importantly, no convincing association with cancer risk has been reported (60). However, the total number of cases of these inborn errors is small, and frequently diagnosis is made in childhood and the patients are lost to follow-up before the age of greatest cancer risk. On the other hand, the redundancy of three substrate sources (arginine, proline, and glutamate) as well as the duplication of isozymes (P5CS-L and P5CS-S; PYCR1 and PYCR2) is a prime example of epistasis that may account for the difficulty for convincing identification of phenotypes.

Although 4-hydroxyproline, the proline congener abundant in mammalian species, is structurally similar to proline, its metabolism is distinctly different (2). A critical contributor to the physical structure of organisms, hydroxyproline is not found in species before the evolution of metazoans. Preformed hydroxyproline is not incorporated into protein presumably because all the triplet codons were occupied (2, 52). Instead, it is formed by the posttranslational hydroxylation of proline in proteins (2). Since hydroxyproline is not recycled for protein synthesis, its degradation continues down to 2- and 3-carbon compounds. The degradative pathway, however, shares some of the enzymes metabolizing proline (103). The initial step is catalyzed by hydroxyproline oxidase (*PRODH2*), an enzyme encoded by a gene distinct from that for proline oxidase (*PRODH*) and with little overlap in substrate utilization (91). In contrast, in the second step of their degradation, hydroxyproline and proline share the same enzyme, i.e., P5C dehydrogenase (103). It is interesting that P5C reductase from animals not only converts P5C back to proline for protein synthesis, but also can reduce OH-P5C to hydroxyproline even though product hydroxyproline is not reused for protein synthesis (3). P5C reductase in prokaryotes, however, does not have this activity for OH-P5C (3).

The potential role of the proline metabolic systems as a redox-regulatory system was recognized in the early 1980s (87). During the 1960s,

Elijah Adams noticed that P5C is not only the immediate product of proline degradation, but also its immediate biosynthetic precursor (1). He pointed out that this was very unusual in amino acid metabolism. Our interest in proline metabolism led us to seek several sources of P5C. We made L-P5C enzymatically using partially purified OAT (99). Another source was the commercially available DL-P5C-3, 4, dinitrophenylhydrazone (74, 113). Using P5C from either source, we found that it transferred oxidizing potential rapidly into cells and markedly stimulated the activity of the pentose phosphate shunt (89, 90). The level of stimulation was greater than with any metabolic intermediate and approached that produced by methylene blue. Using reconstituted systems, the enzymatic transfer of redox equivalents between mitochondria and cytosol was demonstrated, leading to the formulation of the proline cycle. The proline cycle defines the redox functions of proline metabolism as the transfer of reducing potential into mitochondria and oxidizing potential out of mitochondria by the cycling of proline and P5C (32, 33). The role of these redox transfers, however, remained unclear until recently. Nevertheless, the cycle served as the basis for our understanding of the stress responses of proline metabolism during carcinogenesis and cancer progression (92). The redox functions have been convincingly demonstrated in cultured cells from patients with inborn errors (95) as well as in plants (75).

## COLLAGEN METABOLISM

### Collagen: A Metabolic Reservoir

The potential role of collagen as a storage reservoir for amino acids in a fashion paralleling glycogen for glucose and adipose tissue for fatty acids has been previously mentioned; the special functions of proline and also hydroxyproline as metabolic substrates are outlined above. But there are distinctions and also parallels in the metabolism of these two imino acids. Proline is proteinogenic and is



available from the digestion of dietary proteins, endogenous biosynthesis from both glutamate and ornithine and recycling from proteolysis (1). Free hydroxyproline, on the other hand, is not reused for protein synthesis (see above) and was thought to be degraded terminally to glyoxylate and pyruvate (2). Recent studies suggest that hydroxyproline, like proline, can be recycled via a redox shuttle (13). We are only beginning to investigate the participation of hydroxyproline in redox and metabolic regulation. For this review, we limit our discussion to the special metabolic features for proline.

The aforementioned metabolic pathways providing endogenous nutrition in the microenvironment require a mobilizable source of this amino acid, especially under conditions of nutrient stress, when the usual sources of substrate proline and its amino acid precursors are unavailable. This is especially important for wound healing physiologically and for tumor progression pathophysiologically. For both of these conditions, there is a source in the large quantities of extracellular matrix (ECM) in the microenvironment (15, 81). Collagens make up 80% of ECM, and type I collagen is the most abundant protein in the body (21). It not only is the major part of ECM but also comprises 90% to 95% of the protein in connective tissue and is the organic part of bone (108). Although glycine is also abundant in collagen, it is metabolically promiscuous and not amenable to specific regulation. In contrast, proline and hydroxyproline, which together make up 20% to 25% of the residues of collagen, have a regulated and directed metabolism because of their unique structures. In this context, collagen can serve as a microenvironmental reservoir of mobilizable substrate. This is shown schematically in **Figure 2**.

Collagen in ECM and bone is constantly remodeled. In bone, where it has been extensively studied, collagen is laid down by osteoblasts. Its degradation, on the other hand, is mediated by osteoclasts, a differentiated macrophage with a profile of enzymes that resorb bone and digest the organic matrix (102). The process of

collagen turnover has been studied in tissue culture using a fibroblast model (42). Interestingly, a large percentage of the newly synthesized collagen is degraded before it is secreted from the cell to form the helical structure of collagen fibrils. Since the hydroxyproline derived from this degradation of nascent collagen is not reused for protein synthesis, it is tempting to speculate that free hydroxyproline, which can only be endogenously synthesized after peptide linkage in collagen, is the desired product from this degradation. However, no unique function for free hydroxyproline has yet been identified. Nevertheless, it is noteworthy that hydroxyproline oxidase, catalyzing the first step in hydroxyproline degradation, like proline oxidase, is encoded by a p53-induced gene. Furthermore, hydroxyproline and 3-OH pyrroline-5-carboxylate participate in a metabolic cycle much like that for proline and pyrroline-5-carboxylate (13).

### Activation of Matrix Metalloproteinases

The MMP family of proteases is markedly activated during wound healing and in many physiologic and pathologic processes (79, 102, 115). Especially relevant is the role of MMPs in cancer progression (10, 55, 58, 78). These enzymes have been characterized and their functions outlined in a recent review (115). Early on, the marked increase in MMP activity during tumor invasion and progression was thought to be critical to the breakdown of tissue structure for tumor invasion (15). In both tissue culture and animal models, the inhibition of MMP activity by broad-spectrum inhibitors, e.g., batimastat, had a marked inhibitory effect on cellular proliferation and tumor growth (29). However, when these preclinical findings were tested in clinical trials involving various human tumors, the results were disappointing (14). The lack of efficacy was thought to be due to complexities of tumor progression and metastasis. The studies were performed in patients with considerable variation in tumor progression and metastasis,

and it is likely that blockade of MMPs may be effective only at certain as yet undefined stages of tumor development. An important concept supported by convincing evidence is that the removal of physical constraints by MMPs is only a minor component of its effect on tumors. More important is the generation or release of *de novo* and preformed bioactive factors that stimulate growth (51). The release of preformed TGF  $\beta$  from its binding to ECM is one such example. Although these are important considerations, another aspect of MMP-mediated collagenolysis may be metabolic; the release of novel amino acid substrates in the microenvironment during nutrient stress has not been recognized. That this is potentially an important source of substrates is supported by the finding of intracellular uptake of collagen fragments and its degradation by uPARAP/Endo 180 (16). Studies of this metabolic pathway showed that fragments are taken up into lysosomes and amino acids released. Thus, like the activation of autophagy under conditions of nutrient stress, MMP activation mediates the utilization of a local source of substrate, collagen, under conditions of nutrient stress (57). We have designated this process as *ecophagy*, that is, the consumption of extracellular proteins in the cellular environment. There is abundant evidence that collagen is degraded—with inflammation (15, 86), during hypoxia (57), and in experimental models of tumorigenesis (70). There have been no measurements of *in situ* metabolism of free proline and/or hydroxyproline due to experimental difficulties. Nevertheless, recent studies using proteomic technologies report increased proline and hydroxyproline consumption in patients with metastatic prostate tumors (11).

### Prolidase

An important enzyme in releasing proline and hydroxyproline for metabolism from collagenolysis is prolidase (69). Although structurally unrelated to the MMP family, prolidase plays an important role in the complete degradation of the collagens in ECM.

Collagen fragments are taken up into lysosomes and, with the action of uPARAP/Endo 180, cathepsins, and peptidases, are degraded, and amino acids are released for metabolism and for protein synthesis. But dipeptides containing proline or hydroxyproline require their own special imidodipeptidase (69). Depending on whether the imino acid is the amino or carboxyl terminus, prolinase and prolidase, respectively, hydrolyze the peptide bond. With the amino acid sequences found in collagen, the most abundant imidodipeptides are substrates for prolidase. Thus, if the release of free proline (or hydroxyproline) contributes to the ECM turnover in physiologic or pathophysiologic states, prolidase would catalyze a critical step.

An experiment in nature exists in that a group of international patients with inborn deficiency of prolidase has been identified (67, 68). A prominent finding in these patients with prolidase deficiency is large ulcers due to defective wound healing. In addition, these patients have immunodeficiencies. Evidence linking these deficiencies with cancer risk, however, is lacking. But the total number of patients described globally would not constitute enough numbers to show increased cancer risk. Furthermore, the defects are identified in children, and there is limited life expectancy. The patients frequently die during the fourth or fifth decade of life due to susceptibility to infections. Although it has been suggested that collagen synthesis is defective in the nonhealing wounds in these patients because of a deficiency of recycled proline, just how this source of stress substrate fits into collagen remodeling and immune regulation remains unclear. Nevertheless, it is interesting that prolidase can be regulated by a number of factors including integrins (82), IGF-1 (76) at the level of synthesis, and by nitric oxide at the level of serine/threonine phosphorylation (100). Several laboratories are interested in developing prolidase knockout mice in order to obtain experimental models to test carcinogenesis or cancer metastasis. Tumor cells with prolidase knocked out would be useful as xenografts.



## PROLINE AS STRESS SUBSTRATE

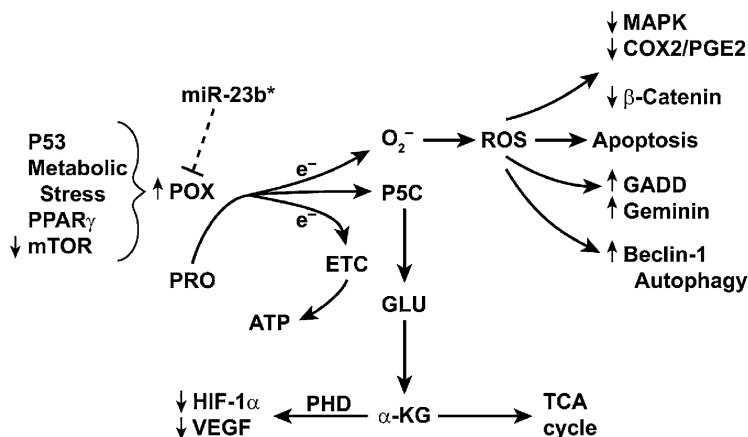
### Stress Inducers of Proline Oxidase

The initial discoveries of proline metabolic biochemistry were made during the 1950s and 1960s (1). The emphasis was on models and paradigms of metabolism serving the whole organism. Studies in mammalian species including humans focused on homeostasis and were strongly influenced by ideas developed from endocrinology and metabolism (87). But the paradigm-shifting discovery in proline metabolism was the serendipitous finding in a screening study of the genes induced by the cancer suppressor, p53 (94). Using an adenoviral vector to express wild-type p53 in p53 mutant cells and monitoring the genes expressed with serial analysis of gene expression (SAGE), Polyak et al. (in Bert Vogelstein's lab; 94) found that POX/PRODH was markedly upregulated by p53. Of 7202 genes monitored, only 14 genes were induced greater than sevenfold, and POX/PRODH was one of the 14 and designated as p53-induced gene 6 (PIG6).

Yu, Hu, and colleagues developed a tet-off POX expression plasmid and obtained stable transfectants in DLD-1 colorectal cancer

cells (23). An important initial finding was that POX/PRODH generated proline-dependent reactive oxygen species (ROS) (23). That the overexpression of POX/PRODH and generation of ROS could initiate apoptosis was shown independently by several laboratories (37, 39, 61, 71). Using the stably transfected DLD-tet-off-POX cells, Hu et al. documented POX/PRODH expression and activity and showed induction of proline-dependent apoptosis independent of p53; the mediator of the effect was ROS (23, 61). Thus, it was established that genotoxic stress, through the p53-mediated pathway, activated POX/PRODH, which played an important role in the onset of apoptosis. The details of this effect in carcinogenesis are described in a following section.

The discovery that POX/PRODH played a role in apoptosis was the beginning of a new era in research in the proline metabolic pathway (Figure 3). In contrast to earlier studies emphasizing homeostasis and whole-organism regulation, the new paradigm suggested that the proline metabolic pathway was mobilized primarily under conditions of stress, was localized to a microenvironment, and could use as substrate not only free cellular proline but also



**Figure 3**

Responses of POX to various stress stimuli and their metabolic consequences. ATP, adenosine triphosphate; COX2, cyclooxygenase-2; ETC, electron transport chain; GADD, growth arrest DNA damage; GLU, glutamic acid; HIF-1 $\alpha$ , hypoxia-inducible factor-1 $\alpha$ ; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; PPAR $\gamma$ , peroxisomal proliferator-activated receptor gamma; POX, proline oxidase; PRO, proline; Pyr, pyruvate; ROS, reactive oxygen species.

could tap the reservoir released from collagen. Although p53 and POX were first linked in the context of apoptosis and programmed cell death, recent work suggests that p53 is also an important regulator of metabolism (7, 8). Thus, the linkage of p53 and POX/PRODH holds for both of these areas of cell regulation. POX/PRODH and proline were previously well recognized as a substrate source for bioenergetics and carbon exchange (1, 87), but only recently as mediators of signaling (60, 62, 63). P53, on the other hand, which was discovered as a regulatory protein for programmed cell death and as a cancer suppressor protein (107), is now recognized to have an important role in regulating metabolism (7, 107). Thus, this dual role applies to both these pathways.

A contribution to the above paradigm was made by Pandhare and coworkers, who discovered the involvement of peroxisomal proliferator-activated receptor gamma (PPAR $\gamma$ ) (83). They systematically screened transcriptional factors using a POX promoter, and although a number of well-recognized factors (including AP-1 and NF- $\kappa$ B) produced modest stimulation of POX expression, PPAR $\gamma$  and its thiazolidinedione ligands, used to treat type 2 diabetes (54), produced a marked effect. Expression of PPAR $\gamma$  and exposure to troglitazone stimulated the POX promoter more than tenfold. That this was due to the ligand-dependent binding of PPAR $\gamma$  to its binding site on the POX promoter was established using electrophoretic mobility shift and chromosomal immunoprecipitation assays. This finding revealed that the participation of POX/PRODH and proline metabolism was not limited only to one set of stress conditions, i.e., genotoxic stress. A discussion of the complexities of PPAR $\gamma$  is not within the scope of this review, but briefly, it plays many roles in metabolism—it is produced by adipocytes but primarily is a response to inflammatory stress. In addition, its functional role involves the participation of several factors including retinoid X receptor, and PPAR $\gamma$  coactivator-1 allows it to respond to a number of regulators (80, 98). Thus, multiple levels of regulation, its role in fat

metabolism and in diabetes, and the interaction with inflammation make this a highly interesting interaction with the POX/PRODH pathway. Our findings using colorectal cancer cells were corroborated by workers using lung cancer cells (48). Although this interaction has been established, its clinical relevance remains to be identified.

## Metabolic Consequences

It is not surprising that the proline metabolic pathway also is linked to metabolic or nutrient stress. Although the two previously mentioned responses, i.e., to genotoxic stress and inflammatory stress, may occur independently of nutrient stress, the mobilization of proline from the microenvironment may be especially important when other nutrients or substrates are depleted. Of course, hypoxia is frequently a concomitant of nutrient depletion—when tissues are deprived of their blood supply (85). This can occur during coronary occlusion, cerebrovascular accidents, wound healing, or the decreased blood supply for an invading tumor. In fact, nutrients are more severely depleted than oxygen because oxygen can diffuse through tissue with greater efficiency than nutrients. Thus, it can be assumed that all the circulating nutrients, i.e., glucose, glutamine, fatty acids, and circulating amino acids, would be at low supply. We tested the effect of glucose deprivation on the regulation of POX and found that POX activity indeed increases (84). Since the response to nutrient and energy deprivation is mediated through the mTOR pathway, we examined the effects of this pathway. As expected, treatment of cells with rapamycin at low concentrations markedly induced POX (84). Similarly, amino-imidazolecarboxamide ribonucleoside, an analogue of AMP, also upregulated POX activity. Thus, inhibitors of mTOR signaling, nutrient and/or energy depletion, stimulated POX activity. Blockade of TOR signaling stimulates autophagy and promotes survival by an energy-dependent process (56). We monitored cellular ATP levels in cells exposed to rapamycin and found that ATP levels were

maintained after an initial depression (84). However, if POX induction were blocked by treatment with POX siRNAs, ATP levels rapidly and markedly decreased. Thus, it appears that POX induction plays a role in maintaining cellular energy for survival.

But what is the metabolic mechanism by which ATP is maintained? Certainly the electrons from proline donated to the electron transport chain can generate ATP, albeit inefficiently, and the degradation of proline will produce  $\alpha$ KG, an intermediate in the TCA cycle. However, under pathophysiologic conditions of nutrient stress, the TCA cycle would be operating inefficiently owing to concomitant hypoxia and glucose depletion. In their classic studies of glucose metabolism through the pentose phosphate pathway (PPP), Eggleston & Krebs (24) pointed out that the critical regulation of the oxidative arm of the PPP would be the regeneration of  $\text{NADP}^+$ , the rate-controlling cofactor for glucose-6-phosphate dehydrogenase. To test this possibility, we used the model DLD-POX cells and found that whereas expression of POX had only modest effects on glycolysis, it increased the flux through the PPP by more than fivefold (84). This occurred at all levels of glucose tested. We further showed that the maintenance of ATP under rapamycin treatment not only required the induction of POX, but also required the functioning of the PPP as ATP levels were inhibited by dehydroepiandrosterone, an inhibitor of glucose-6-phosphate dehydrogenase (28). Thus, in response to downregulation of the mTOR pathway, proline was metabolized as a source of energy, and this effect included activation of glucose metabolism through the PPP.

The bivalent function for POX with divergent metabolic consequences deserves elaboration. Proline-derived electrons are transferred to the enzyme-bound flavin adenine dinucleotide (FAD). The electrons can be transferred into site II of the electron transport chain through cytochrome c. Tanner's lab, however, has shown that the FAD is directly accessible to solvent oxygen; the

electrons can directly reduce oxygen to yield superoxide (111). Their work showed that in vitro translated and purified monofunctional POX from *Thermus thermophilus* could produce superoxide from proline. Furthermore, they showed that an adjacent  $\alpha$ -helix could shield FAD from oxygen. On the basis of these findings, the authors proposed that proline-derived electrons can either reduce oxygen to form superoxide or can enter the electron transport chain to generate ATP (111). Obviously, the identification of a mechanism switching between superoxide and ATP would be of great interest.

P5C produced by proline oxidase emerges from mitochondria and is converted back to proline by P5C reductase, completing the proline cycle. There are two isozymes of P5C reductase, PYCR1 and PYCR2. In a lymphoblastoid cell line, PYCR1 is missing, making the cells partial proline auxotrophs (38, 64). PYCR1 has low affinity for pyridine nucleotide but has high  $V_{\text{max}}$  for proline formation. PYCR2, in contrast, has high affinity for NADPH but relatively lower  $V_{\text{max}}$  for proline synthesis (64). The identification of PYCR1 mutations in patients with cutis laxa is of great interest because these patients have skeletal and neurodevelopmental abnormalities. Their cultured fibroblasts are more sensitive to hydrogen peroxide-induced toxicity, a finding corroborating the redox functions of proline metabolism (95). In addition, cells with PYCR1 deficiency show mitochondrial abnormalities, pointing to the overall importance of the pathway. The structure of human PYCR1 has been determined by X-ray crystallography, and although homodimers are catalytic, the native enzyme exists as a decamer consisting of five homodimers (26, 72). The structure resembles that seen in proteins with transport or chaperone functions. Thus, the function of P5C as substrate for this protein is of considerable interest. P5C also can be converted to glutamate by P5C dehydrogenase and then to  $\alpha$ KG. The  $\alpha$ KG from mitochondrial POX is not only substrate for the TCA cycle but also as a regulatory molecule (see below).

## PROLINE METABOLISM AND CANCER

The resurgence of interest in tumor metabolism was in part due to the discoveries in cancer genomics that showed multitudinous mutations in any single tumor. A catalog of these genomes revealed over 100 mutated genes in any one tumor (26). Although some of these genes have been relegated to the status of passenger genes, and others are designated driver genes (30), the inescapable conclusion is that pathways rather than specific genes are mutated and selected by the tumor for evading stress situations or host defense mechanisms, thereby allowing its survival. Importantly, the mutated genes constitute a genetic record not only of proliferation signals but also of adaptations for survival under various conditions imposed temporally and spatially by anatomic distortions, host defenses, and the logistics supporting rapid neoplastic growth (105).

Recently, the idea that cancer stem cells underlie carcinogenesis has become pervasive (47). The hypothesis holds that a few cells with some or all of the necessary mutated genes retain their stemness of differentiation potential to express any or all of these mutated genes (31). Thus, depending on the stresses imposed by the microenvironment, by host defenses, or by cytotoxic therapy, stem cells can survive the stress or evade the therapeutic insult. But the survival of cells through the metabolic gauntlets drives the selection. The pioneering work of Otto Warburg (109) has received considerable attention. His Nobel Prize-winning discovery can be summed up as the adaptation or even “addiction” of tumor cells to glycolysis even in the presence of oxygen (105). Of course, these characteristics represent the metabolic features of a “successful” tumor cell, proliferating at a rapid rate. These concepts are helpful in understanding observed changes in proline metabolism during carcinogenesis.

### Effect of POX on Cancers

Although we have briefly discussed the mobilization of the proline metabolic system as

a stress response, we must now apply these responses specifically to carcinogenesis. Early studies on the enzymes of proline metabolism focused on the endogenous biosynthesis of proline as a proteinogenic amino acid. Comparison of enzyme-specific activities showed that some of the enzymes in the pathway for synthesis were higher in tumors than in corresponding normal tissues (35). These differences, however, were modest and could not be generalized to all neoplastic tissues. Because of these inconsistencies, further characterization of these enzymes in various tumors was not pursued. During the genomics era of cancer research, these genes were classified as housekeepers.

Nevertheless, by the early 1980s, the special, regulatory functions of proline metabolism were recognized (87). But the niche for these metabolic functions remained largely unknown until the discovery that the gene encoding POX/PRODH is p53 induced (94). Because p53 is a critical responder to genotoxic stress and is recognized as a universal tumor suppressor gene, the induction of POX/PRODH by p53 led to the recognition that one of the special functions of proline metabolism is in carcinogenesis (88).

POX/PRODH expression responds not only to genotoxic stress but also to inflammatory stress and metabolic stress (see above). PPAR $\gamma$  is of special interest because its regulation of transcription requires the cooperation of a number of factors, including retinoid X receptor, PPAR $\gamma$  coactivator-1, and P300/CEBP (34, 80, 98). Thus, the regulation of POX/PRODH expression mediated by this transcriptional factor can be modulated by a number of mechanisms. We and others showed that the apoptosis induced by PPAR $\gamma$  is mediated by its induction of POX/PRODH and the generation of proline-dependent ROS by POX/PRODH (48, 83). These findings are of considerable interest because of the decreased incidence of certain cancers, e.g., lung, in diabetic patients treated with thiazolidinediones. Clinical trials using pioglitazone as a chemopreventive are under way.

Since POX/PRODH is upregulated in response to three types of stress, i.e., genotoxic stress, inflammatory stress, and metabolic stress, and the enzymatic activity catalyzed by POX/PRODH yields a number of products, how and under what conditions is this activity relevant to cancer (**Figure 3**)? First, electrons from proline are transferred to a flavine adenine dinucleotide at the enzyme active site. These electrons can either directly reduce oxygen to form superoxide which is converted to hydrogen peroxide by superoxide dismutase (SOD) 2 or they are transferred to cytochrome c into the electron transport chain to produce ATP (111). The other product, P5C, has a number of metabolic fates. Pyrroline-5-carboxylate dehydrogenase converts P5C to glutamate, which can then be converted to  $\alpha$ KG. Since  $\alpha$ KG is not only a central substrate for the TCA cycle but also a critical substrate for the prolyl hydroxylation of HIF-1 $\alpha$ , a change in the availability of  $\alpha$ KG has been proposed as a regulator of HIF-1 signaling (36, 50). Finally, P5C is converted back to proline by (PYCR1/2) (**Figure 1**). Together with POX/PRODH, PYCR1/2 catalyze the proline cycle, which can be used to regulate redox in the cell (87).

In the context of signaling for apoptosis and programmed cell death, the generation of superoxide by POX/PRODH was an important finding (61). A number of the downstream effects of POX/PRODH were shown to be abrogated by the coexpression of SOD2. Thus, the activation of both the intrinsic (mitochondrial) and extrinsic (death receptor) limbs for apoptosis by the expression of POX/PRODH can be blocked, at least in part, by SOD2 (62). Furthermore, the downregulation of MAPK (62) and COX-2/PGE2 (63) signaling all appear to be dependent on the generation of ROS. Is there something special about the POX-mediated generation of ROS? The chemical biology of ROS and its generation from sporadic leakage from the electron transport chain (53) or as a result of enzyme-mediated events from NADPH oxidase (46) and/or xanthine oxidase (9) are beyond the scope of this review. However, the

fact that POX is a mitochondrial membrane-bound enzyme that is highly regulated has advantages over the other aforementioned sources of ROS. It is likely that the leakage of electrons from the electron transport chain is sporadic, and the scavenging by small molecules and enzyme-mediated inactivation of these radicals protect against their damaging effects (53). It has been proposed that NADPH oxidase can be regulated to produce superoxide and that this is a source of ROS signaling (12). This may be the case for growth factor-mediated signaling that occurs primarily at the cell membrane, the location of NADPH oxidase (12). However, it may be that POX/PRODH is a regulated mitochondrial source of ROS that may be directed to controlling mitochondrial or intrinsic apoptosis.

The electrons from proline can also be donated to electron transport chain via cytochrome c to produce ATP (1, 2, 87). This certainly occurs under conditions of nutrient deprivation, when POX activity is markedly increased. In addition, with rapamycin inhibition of mTOR, the maintenance of cellular ATP appears to be proline dependent (84). The generation of ATP from proline is maintained in part by the metabolic interlock between the proline cycle and the PPP (**Figure 2**) (32, 33, 87). Under conditions of nutrient stress, when glucose is inadequate to maintain ATP through glycolysis, glucose can be completely converted to CO<sub>2</sub> by the oxidative and nonoxidative arms of the pentose phosphate shunt. Reducing potential in the form of NADPH is transferred into mitochondria for ATP generation by the cycling of P5C and proline. When POX/PRODH is expressed, the PPP is increased more than fivefold (88), and ATP generation accompanying this activation can be markedly inhibited by dehydroepiandrosterone, an inhibitor of glucose-6-phosphate dehydrogenase, the rate-limiting enzyme of the oxidative arm of the PPP (28). Of course, the proline cycle is made up of three enzymes, POX/PRODH and PYCR1/2. The importance of this cycle in maintaining cellular redox states was recently demonstrated in patients with an inborn deficiency



of PYCR1. Loss of the enzyme results in a syndrome constellation that includes cutis laxa, characteristic facies, bony and neurological abnormalities, and progeroid features. Fibroblasts from these patients show markedly decreased resistance to the toxicity from exogenous  $H_2O_2$  (95).

Recent work by investigators has highlighted the increase in activity of the PPP in cancer cells under various conditions. TIGAR induced by p53 may increase the generation of ribose by the PPP to increase the salvage and de novo pathways for nucleotide synthesis (7). The regulation of flux through the oxidative arm of the PPP is regulated by NADP/NADPH ratios, and appropriate coupling with the PPP can generate ATP—producing reducing potential (87) if the appropriate redox shuttle is functioning. Thus, the induction of POX/PRODH by p53 and by nutrient deprivation could generate ATP in the absence of a large glycolytic flux and also could increase the flux from glucose-6-phosphate to ribose-5-phosphate for the synthesis of nucleotides (117).

In cultured lymphoma cells, increased glutaminolysis has been associated with the marked increase in glycolytic flux (17). Dang et al. (18) and Vander Heiden et al. (105) have emphasized the importance of these metabolic adaptations to the generation of cell mass. Indeed, glutamine is necessary as nitrogen donor and is a necessary substrate for the first step in de novo purine synthesis (114). By the time the tumor has progressed to the generation of cell mass, it is likely that the gauntlet of metabolic stress has been successfully traversed. Thus, in this state, bioenergetics is no longer an obstacle. Neovascularization has taken place, and the supply of glucose and glutamine is amply provided by new blood vessels.

The function of downstream  $\alpha$ KG is also of interest. Since prolyl hydroxylase is a dioxygenase that uses  $\alpha$ KG as substrate (36), the hydroxylation of HIF-1 $\alpha$  by the family of prolyl hydroxylases can be affected by the concentration of  $\alpha$ KG (50, 65), and hydroxylated HIF-1 $\alpha$  binds to Von Hippel-Lindau protein and is directed to proteasomal degradation.

Thus, mitochondrial tumor suppressors (component enzymes of the TCA cycle) have been identified. Because P5C is sequentially dehydrogenated to glutamate and  $\alpha$ KG, increased POX/PRODH would augment the degradation of HIF-1 $\alpha$ . Increased prolyl hydroxylation mediated by  $\alpha$ KG would lead to increased HIF-1 $\alpha$  degradation. This function was observed in recent experiments demonstrating that overexpression of POX/PRODH in DLD-POX cells resulted in increased levels of  $\alpha$ KG, decreased levels of HIF-1 $\alpha$ , and decreased VEGF (60). Whether this regulatory signaling occurs with POX/PRODH levels regulated by physiologic or pathophysiologic stress signaling requires further studies.

Another area that needs additional elaboration pertains to metabolic stress and/or nutrient deprivation. Under conditions of hypovascularity, hypoxia and nutrient deprivation ensue, and this would apply not only to glucose but also to all nutrients usually delivered through the circulation. This would include not only glucose but also circulating amino acids, and in particular, glutamine, which is the principal currency for transfer of amino acid nitrogen between muscle and liver and kidney (27). Similarly, the delivery of fatty acids would be limited. We have proposed that ecophagy, the MMP-mediated degradation of ECM in the microenvironment of a tumor cell, may be the first response for evading death due to nutritional starvation (88). Following ecophagy, autophagy, the degradation of cellular constituents, is activated as a source of bioenergetics for survival. Finally, if autophagy is inadequate for survival, apoptosis ensues. Of course, even apoptosis is an orderly, energy-requiring program for recycling of cellular constituents.

## POX as Tumor Suppressor

But do these mechanisms that have been demonstrated in tissue culture participate as in vivo mechanisms during carcinogenesis? We attempted to translate these effects to an in vivo model in mice. The DLD-1 colorectal cancer



cells readily form xenograft tumors when injected into the flanks of immunodeficient mice (60). DLD-tet-off-POX cells will not express POX/PRODH when mice are given doxycycline in their drinking water. When doxycycline is withdrawn, POX/PRODH will be overexpressed in these cells. We first suppressed POX/PRODH expression and allowed the engrafted tumors to establish and grow to 100 cu mm. At that time, doxycycline was removed, and POX/PRODH expression was documented in the tumors. With established tumors, expression of POX/PRODH had little effect on further growth. By contrast, when cells were injected into animals with or without doxycycline in their drinking water, the expression of POX completely suppressed the engraftment and growth of tumors (60). After two weeks, 16 of the 20 animals without doxycycline still had no palpable tumors. Eight of the animals were then given doxycycline-containing drinking water to suppress POX and they readily grew tumors, showing that the tumor cells were indeed present and viable but growth had been suppressed by POX expression. These findings further suggested that although apoptosis was the model in the tissue culture system, suppression of growth appeared to be the dominant effect *in vivo* (60). These observations clearly showed that the effect of POX could be translated into an *in vivo* animal model. Importantly, they strongly suggest that POX/PRODH functions as a tumor-suppressor protein.

The consideration of POX/PRODH as a tumor suppressor requires evidence that human tumors have evaded this suppressor, *i.e.*, that human tumors have lost or downregulated this protein. In order to examine this possibility, we devised an immunohistochemical assay for POX/PRODH and examined 97 human tumors from a variety of tissue sources together with matched normal tissues from the same patient. For most cases, the normal controls were from adjacent tissues. From these diverse tumors, 56% had decreased levels of POX expression, a finding that was of borderline significance. However, when the tumors ( $N =$

37 pairs) of the digestive tract (colon, rectum, stomach, pancreas, and esophagus) were considered, 78% of the tumors had markedly decreased or undetectable POX as compared to normal tissues (60). From this source as well as two additional sources, 26 renal carcinomas were compared to normal tissues, and 85% of the tumors had markedly decreased or undetectable POX (59). Thus, from human tumors monitored by immunohistochemistry, POX expression was markedly decreased in a variety of human tumors, but particularly those of the digestive tract and kidney. For the latter, it was previously reported that POX mRNA was decreased in renal tumors, consistent with a robust decrease in POX protein. Thus, the downregulation of POX in human cancers has been demonstrated, and together with the extensive data from *in vitro* studies, the tumor suppressor function of POX/PRODH has been firmly established.

The classic tumor-suppressor protein, p53, fits the Knudsen model of single nucleotide polymorphisms in one allele and somatic mutation in the other allele, leading to marked reduction or loss of activity (49). Thus, we examined the POX gene, seeking mutations in the tumors. Although we found some single nucleotide polymorphisms, these did not correlate with loss of activity; mutations in tumors with loss of POX/PRODH expression were not evident. Epigenetic mechanisms were another possibility, and we screened the POX/PRODH promoter and coding sequence for CPG islands but found no differences between normal and colorectal tumor tissues in methylation. Although these have not been ruled out, common epigenetic mechanisms did not cause the differences between normal and tumor expression of POX/PRODH.

### POX Regulated by MicroRNA

We found a specific microRNA, miR-23b\* (the asterisk denotes the less predominant form of the expressed precursor), that potently suppresses the expression of POX/PRODH. It produces its effect primarily at the level of

protein translation, a feature common to many miRNAs (59). Not only does mimic RNA of miR-23b\* suppress POX/PRODH expression, its antagomir also increases POX/PRODH expression, at least in tumor cells with low POX/PRODH expression. The binding of miR-23b\* to the 3'-UTR of the POX/PRODH gene was shown using a luciferase assay. To make the critical correlation of this miR-23b\* to POX/PRODH for human cancer, we obtained frozen tumors and paired normal tissues from 16 clear cell renal carcinomas. The choice of a histologically defined tumor helped minimize biologic diversity. From this group, 13 of the 16 had markedly decreased POX/PRODH expression as monitored by Western analyses. Furthermore, the tumors had increased levels of miR-23b\*. Importantly, there was an inverse relationship between the levels of the latter and expression of the former. In a statistical test, the inverse relationship was significant at the level of  $p < 0.001$ . The final test was to examine the expression of miR-23b\* using in situ hybridization, which showed that the tumor tissue had markedly increased expression compared to normal kidney tissues. Thus, not only is POX/PRODH a tumor suppressor, but also the mechanism of its downregulation has been established as due to miR-23b\* (59).

This relationship was further examined by monitoring miR-23b\*-mediated up-regulation of POX/PRODH and its functional effects. Using the antagomir for miR-23b\*, we increased POX/PRODH expression in a number of colorectal cancer cells. Importantly, accompanying the increase in POX/PRODH expression was a decrease in cell growth and augmentation of the blockade at the S/G2 checkpoint. The effect of POX/PRODH on levels of HIF-1 $\alpha$  was reproduced by manipulating miR-23b\* (see above). MiR-23b\* mimics not only decreased the induction of POX/PRODH but also modulated the decrease in HIF-1 $\alpha$ . This was especially relevant because the HIF-1 $\alpha$  signaling system plays a special role in renal carcinogenesis. Thus, the POX/PRODH renal suppressor as controlled by miR-23b\* and its relationship

to HIF-1 $\alpha$  may be of special clinical significance (59).

As mentioned above, the induction of POX/PRODH by an adipocyte-derived transcriptional factor, PPAR $\gamma$ , and by its ligands, the thiazolidinediones, frequently used in type 2 diabetes mellitus, are of considerable interest (83, 93). This is especially true since recent studies have emphasized the level of oxidized low-density lipoprotein (oxLDL) as the critical factor in atherogenesis (43). The epidemiologic relationship of obesity and increased levels of oxLDL with increased cancer risk has been established (20). But the molecular mechanisms responsible for this relationship remain almost totally unknown. Thus, we undertook studies using a tissue culture model to elucidate possible links between oxLDL and mechanisms of carcinogenesis (118). First, we established that treatment of colorectal cancer cells as well as nonmalignant endothelial cells with physiological concentrations of oxLDL markedly increased POX expression. Focusing on the former, we showed that 7-ketocholesterol, an abundant constituent of oxLDL, is a ligand for PPAR $\gamma$ , and activation of PPAR $\gamma$  is the mechanism for the induction of POX/PRODH. Furthermore, oxLDL caused both apoptosis and autophagy. But in contrast to the effects of thiazolidinediones, the effect of oxLDL on apoptosis was not blocked by knockdown of POX/PRODH. Presumably with oxLDL, there are POX-dependent and POX-independent mechanisms for inducing apoptosis. By contrast, the oxLDL induction of the conversion of LC3-I to LC3-II, hallmarks for the onset of autophagy (77), was markedly decreased by POX knockdown. Direct evidence that POX can activate autophagy was provided by the DLD-POX cell model. Expression of POX not only activated the conversion of LC3 but also induced beclin-1 expression. These findings showed that oxLDL acting through PPAR $\gamma$  and POX expression to activate autophagy might be a mechanism for mediating tumor cell survival (118). Whether these in vitro mechanisms apply to the in vivo situation has yet to be ascertained.

## Cancer Metabolic Timeline

With these varied and at times paradoxical effects, a regulated pattern can emerge only if the temporal and spatial stresses impacting the evolving tumor are considered (**Figure 4**). First, inflammatory stress (PPAR $\gamma$ ) occurs under a variety of pathologic conditions, and the activation of MMPs and collagen remodeling is a part of this process. POX/PRODH can utilize the substrates available with the degradation of collagen from activated MMPs and can participate in a process to limit chronic inflammation and stimulate healing. When cells have sustained genotoxic stress with DNA damage, increasing p53 levels will induce POX/PRODH, which at first blocks the cell cycle to optimize repair and to provide alternative sources of substrates, e.g., stimulating the production of ribonucleotides (87, 117), but if the damage is beyond repair, the activated POX/PRODH will produce ROS to initiate apoptosis. If, on the other hand, proliferation signals are locked in by mutations (oncogenes), i.e., RAS, AKT/P13K, and APC, cells will detach from their basement membrane sites and become isolated from their blood supply. With nutrient stress, mTOR is downregulated, thereby increasing POX/PRODH expression to utilize substrates released by MMPs from collagen (ecophagy). Because nutrient stress may precede hypoxia, POX/PRODH may be upregulated before HIF-1 $\alpha$ . In fact, the increase in  $\alpha$ -KG from proline metabolism decreases the level of HIF-1 $\alpha$  (60). This downregulation of HIF-1 $\alpha$  is maintained, however, only as long as substrate proline is available. Upon revascularization, with ample supplies of glucose and glutamine, the tumor enters the phase of rapid growth, when its main goal is to optimize the use of substrates to attain increased cell mass (105). Under these conditions, proline is spared for protein synthesis. Thus, POX/PRODH is downregulated. We have provided evidence for POX/PRODH as a tumor suppressor protein in human tumors. Additionally, recent work from our laboratory has shown that POX/PRODH

expression is downregulated by a specific microRNA, miR-23b\* (59). Because the downregulation of POX/PRODH is a concomitant of the so-called epithelial-mesenchymal transition, its upregulation as a reversal of this process, the mesenchymal-epithelial transition has been proposed to accompany tumor metastases. Investigation into such a possibility has been initiated.

The above scenario, although speculative, allows the integration of all the available findings on the regulation of POX/PRODH and its observed consequences; however, it raises intriguing questions. (a) What is the switch that controls whether proline-derived electrons are used to reduce oxygen and generate superoxide rather than channeled into the electron transport chain to generate ATP? (b) When induced by PPAR $\gamma$ , POX/PRODH generates ROS, but what is the determinant for ROS-dependent autophagy versus apoptosis? (c) What regulates the level of miR-23b\*? (d) What is the molecular mechanism by which mTOR upregulates POX/PRODH? (e) What is the role of hydroxyproline in this process? Is it a redundant system, backing up the system using proline, or does it have special, unique features? These are only a few of the questions that remain to be answered.

## THERAPEUTIC STRATEGIES

Finally, we must focus on how this nutrient pathway can be used in novel therapeutic regimens. Because it is a two-edged sword—a source of energy for survival or a signaling mechanism for programmed cell death—the proline metabolic system as therapy presents formidable challenges. Nevertheless, the opportunity exists for some novel approaches for adjunctive therapy. Another point that must be emphasized is that the metabolism of hydroxyproline, similarly regulated by p53 and PPAR $\gamma$ , has been studied only tangentially. In fact, it has occurred to us that hydroxyproline may be increasingly important because, in contrast to proline, it is not needed for protein

synthesis. But focusing on proline for the time being, we see that ensuring a supply of proline during the period following loss of p53 may be critical to supply an alternative apoptotic mechanism. This could be included in a prevention regimen in the diet. It could be especially important when alternative sources of proline from collagen degradation are being blocked, e.g., with MMP inhibitors. If metabolic events are a contributing mechanism in the preclinical studies, the extrapolation of dietary control to clinical studies may be critical. Finally, the creation of nanoparticles containing an antagomir of miR-23b\* to increase POX/PRODH expression and also containing both proline and hydroxyproline as substrate may be useful after POX/PRODH has been suppressed in tumors. However, with metastatic disease, especially to bone, which is a rich source of collagen, POX/PRODH in tumors growing in bone may be an important target. Blockade of POX/PRODH by proline analogs not incorporated into proteins may inhibit tumor growth.

## SUMMARY

Although the induction of POX/PRODH by p53 was a seminal discovery that led to the consideration of the proline metabolic system in carcinogenesis, a number of other regulatory

mechanisms upregulating POX/PRODH relevant to cancer have been discovered. These include PPAR $\gamma$ , whether activated by thiazolidinediones in colorectal cancer cells or nonsmall-cell lung cancer cells or by oxLDL; downregulation of mTOR signaling by rapamycin; amino-imidazolecarboxamide ribonucleoside; and glucose deprivation. A number of downstream metabolic effectors produced by POX/PRODH have been implicated. ROS, specifically superoxide, is a common denominator for a number of these effects, including the activation of both the intrinsic and extrinsic pathways for apoptosis, upregulation of calcineurin/NFAT signaling, downregulation of MAP kinase signaling, decreased expression of COX-2/PGE2, and Wnt/ $\beta$ -catenin signaling. Through the bivalent channeling of proline-derived electrons into either ROS generation or the electron transport chain, ATP can be produced coupled to the metabolic interlock between the proline cycle and the PPP. In addition, in the presence of lipids, the induction of POX acts as an inducer of autophagy by a not well understood, but ROS-dependent, mechanism. Sequential dehydrogenation of pyrroline-5-carboxylate to glutamate and  $\alpha$ KG provides an important substrate for prolyl hydroxylation of HIF-1 $\alpha$ , through which HIF signaling is downregulated.

## DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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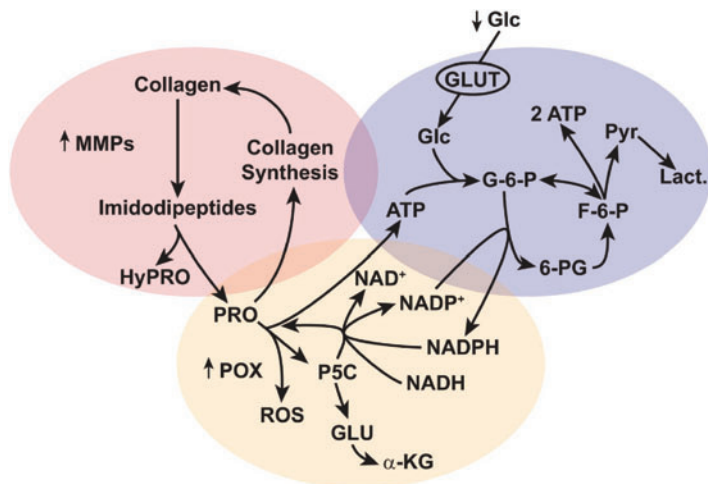
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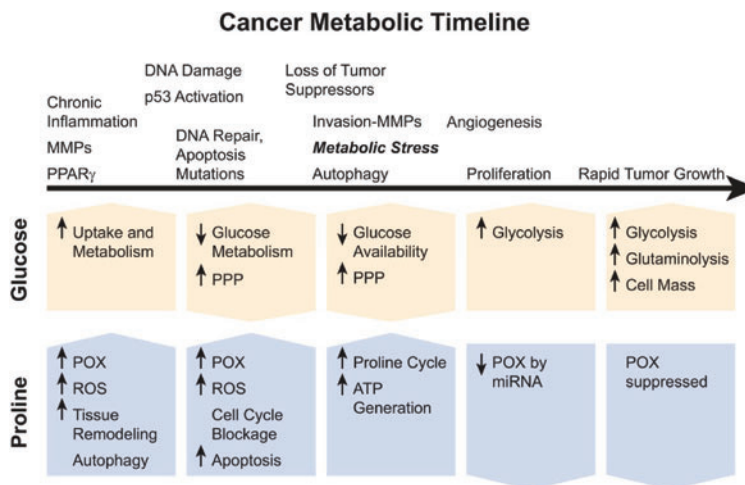
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**Figure 2**

Linkage of extracellular matrix degradation with bioenergetics through proline metabolism in the tumor microenvironment. 6-PG, 6-phosphogluconate; ATP, adenosine triphosphate; F-6-P, fructose-6-phosphate; G-6P, glucose-6-phosphate; Glc, glucose; GLU, glutamic acid; GLUT, glucose transporter; Lact, lactate; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; P5C, pyrroline-5-carboxylate; POX, proline oxidase; PRO, proline; Pyr, pyruvate; ROS, reactive oxygen species.



**Figure 4**

Hypothetical cancer metabolic timeline. The progression of a solid tumor from pretransformation through malignant transformation, metabolic stress, angiogenesis, and rapid proliferation. ATP, adenosine triphosphate; miRNA, microRNA; MMP, matrix metalloproteinases; POX, proline oxidase; PPAR, peroxisomal proliferator-activated receptor; PPP, pentose phosphate pathway; ROS, reactive oxygen species.



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## Errata

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