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The role of poly ADP-ribose synthetase in the cellular regulation of prolyl hydroxylase

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THE ROLE OF POLY ADP-RIBOSE SYNTHETASE IN THE CELLULAR REGULATION OF PROLYL HYDROXYLASE

by

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THESIS

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THE ROLE OF POLY ADP-RIBOSE SYNTHETASE IN CELLULAR REGULATION OF PROLYL HYDROXYLASE

ABSTRACT

Collagen synthesis is a key step in wound healing processes. It can be expected that an understanding of the mechanisms of regulation of collagen synthesis can be used in developing strategies for promoting tissue repair. Prolyl hydroxylase is a major post-translational enzyme in the biosynthesis of collagen. Cellular prolyl hydroxylase activity is correlated with collagen synthesis and is considered an important index of tissue repair, but regulation of prolyl hydroxylase activity is not well understood. The cellular levels of prolyl hydroxylase are modulated by numerous unrelated stimuli: e.g.age, cell proliferation, inflammation, ascorbate, tissue oxygenation and certain superoxide-generating drugs including Bleomycin and paraquat. The only common feature in these conditions is increased cellular levels of oxygen free radicals. Under these conditions, the cells are subject to regulation by the so called "pro-oxidant" state proposed by Cerutti. According to this hypothesis, free radicals induce increased poly ADP-ribosylation (PADPR) of certain nuclear protein resulting in the unmasking of genes and specific gene expression. PADPR is an epigenetic site of regulation of gene expression. The pro-oxidant state and its accompanying biochemical phenomena contribute to cell and tissue differentiation. Inhibition of PADPR has been shown to interfere with the expression of proteins. I am examining the possibility that similar mechanisms are involved

in the regulation of collagen synthesis. If PADPR is involved in regulating cellular levels of prolyl hydroxylase, then PADPR activity should affect the levels of prolyl hydroxylase. In order to verify this hypothesis, I have investigated the pattern of PADPR synthesis in cultures of neo-natal dermal fibroblasts in 5-8 passages. Ascorbate increased the levels of prolyl hydroxylase and PADPR synthetase (PADPRS) and this increase was abolished by inhibitors of PADPRS. Phorbol ester (12-O-tetradecanoylphorbol-13-acetate, PMA) and cholera toxin were also used in elucidating this mechanism. These agents which are known to increase PADPR in the nucleus markedly elevated prolyl hydroxylase activity. This increase was also abolished by PADPRS inhibitors, supporting a role for PADPR in the modulation of prolyl hydroxylase. In addition, PMA and cholera toxin are stimulators of protein kinase C (PKC) and they are involved in the modulation of membrane associated G-protein signalling pathway. In order to examine if PKC and G-protein mediated signalling pathways may be involved in the regulation of prolyl hydroxylase activity, I used pertussis toxin which is known to block the effects of PMA and cholera toxin by stimulating the inhibitory G-protein. It abolished the increase in prolyl hydroxylase activity in presence of PMA, cholera toxin and ascorbate. An inhibitor of PKC also blocked the increased activity of prolyl hydroxylase by the same stimulators. All of these observations suggest that PADPR, PKC and G-protein mediated signalling pathways are involved in the modulation of prolyl hydroxylase. These results explain the increase in prolyl hydroxylase in pathobiological conditions including surgical wound repair and acute inflammation processes.

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Chapter 1. INTRODUCTION

I. Collagen Synthesis

Collagen synthesis occurs in two stages. In the first stage, collagen polypeptides are synthesized by mechanisms common to all proteins. Procollagen polypeptides are synthesized on the membrane-bound ribosomes of collagen-producing cells, just like other secretory proteins. The newly synthesized procollagen polypeptides are released into the lumina of rough endoplasmic reticulum (RER). The initially synthesized peptides undergo extensive enzymatic modification. Inside the RER, these polypeptides are subject to hydroxylation by three specific enzymes: prolyl-4-hydroxylase, prolyl-3-hydroxylase and lysyl-hydroxylase. Prolyl-4-hydroxylase catalyzes the hydroxylation of peptidyl proline to petidyl-4-hydroxyproline, involving approximately 40% of the imino residues. Prolyl-3-hydroxylase catalyzes the post-translational synthesis of 3-hydroxyproline. This hydroxylation only occurs in the X-position of the X-Y-Gly repeat when the Y-position is occupied by 4-hydroxyproline. The number of 3-hydroxyproline residues in type I collagen is very small, varying from one to three residues per chain. The role of the 3-hydroxyproline residues is not well known. Lysyl-hydroxylase catalyzes the formation of hydroxylysine that occurs through hydroxylation of lysine residues in the Y-position of X-Y-Gly repeat. The hydroxylysyl residues also have important functions. They serve as sites of attachmant for carbohydrate units. Hydroxylysine residues are also essential for the formation of

intermolecular collagen crosslinks. Upon completion of intracellular post-translational modifications, the newly synthesized polypeptides of procollagen associate through their carboxy-terminal extension, the assembly is stabilized by the formation of interchain disulfide bonds, and the collagenous polypeptides fold into the triple-helical conformation (1). The triple-helical procollagen molecules are then transported into the extracellular milieu by an energy-requiring process mediated by the microtubules of the cells. After secretion into the extracellular space, post-translational processing is initiated. (see Fig. 1.1). My study is concerned specifically with the regulation of prolyl-4-hydroxylase. This enzyme will be referred to as prolyl hydroxylase in the rest of my thesis.

II. Prolyl Hydroxylase

Prolyl-4-hydroxylase ("prolyl hydroxylase") is an important enzyme in the synthesis of collagen, catalyzing the post-translational hydroxylation of proline residues. 4-hydroxyproline residues account for approximately 40% of all imino residues in collagen and the term hydroxyproline usually implies this form. Hydroxyproline contributes the characteristic stability of the collagen triple helix and as a result of this reaction, newly synthesized procollagen polypeptide chains can be folded into the triple helical conformation. The formation of hydroxyproline only occurs in proline residues that are present in the Y-position of the repeating X-Y-Gly sequence in collagen. Therefore, 4-hydroxy-proline is found only in a 4-Hyp-Gly

sequence. The 4-hydroxy-proline residues have an important function in that their hydroxyl groups stabilize the triple helix of collagen by participating in a hydrogen bonded water bridge (2). This is very important for the survival of collagen because un-hydroxylated collagen is denatured at 25°, but fully hydroxylated collagen is stable at 40°.

Cellular location	Process
Cell membrane receptors	Stimulus for collagen synthesis
Nucleus	Transcription, specific gene expression
Rough endoplasmic reticulum	Translation, procollagen polypeptide synthesis
Lumen of the rough endoplasmic reticulum	Post-translational processing hydroxylation of Pro and Lys
	Glycosylation of Hyl
	Triple helix formation
Golgi vacuoles	Secretion into extracellular space
	Secretion
Extracellular	Procollagen processing Fibrillogenesis Lysyl oxidation in preparation of cross link formation Non-enzymatic reactions of Lys oxidation products to form cross links Non-enzymatic glycosylation

Fig. 1.1: Steps in collagen synthesis

Co-factor and Co-substrate Requirements for the Hydroxylases: All three hydroxylases have similar co-factors and co-substrates. The co-substrates for prolyl hydroxylase are:

(1). Peptidyl proline (-X-Pro-Gly-).

(2). Oxygen molecules: (O_2) which supplies the oxygen atom in the hydroxyl group (3).

(3). α-ketoglutarate: which might act as an allosteric activator in these reactions (4). α-ketoglutarate is oxidatively decarboxylated to succinate in the coupled reaction.

In addition, prolyl hydroxylase requires two co-factors.

(4). Ferrous iron (Fe^{2+}) which serves as the prosthetic group of the enzyme and it is involved in the reductive fixation of O_2. It also participates in generating reducing radicals which reduce the iron prosthetic groups back to Fe^{2+} state after it has been oxidized to Fe^{3+} form after each hydroxylation event, and

(5). Ascorbate (5) provides the electrons to reduce both O_2 and Fe^{3+} back to Fe^{2+} in the enzyme. Ascorbate is also consumed in the reaction.

The cellular levels of prolyl hydroxylase activity can be affected by numerous unrelated factors such as age, cell proliferation, inflammation, ascorbate, tissue oxygenation and certain drugs (6).

Aging: Aging not only has significant effects on the cells with major biological consequences such as: gene mutation, sister chromatid exchanges, chromosomal aberrations, cytotoxcity, carcinogenesis and cellular degeneration (7), but it is also well known that aging affects cellular levels of collagen synthesis (8,9). Many observations show that the metabolism of collagen is markedly different in fetal and adult tissues. Fetal tissues have higher rates of macromolecular turnover consistent with the ongoing processes of tissue reorganization, remodeling and growth (10,11). Fetal repair occurs without inflammation and scar formation (12). In contrast, in adult tissues these processes proceed at very low rates and inflammation is required as a stimulus for collagen synthesis and repair (13). Thus, the biochemical mechanisms that modulate differentiation and macromolecular turnover must be down-regulated in normal adult tissues. Age is also one of the modulating factors in collagen synthesis. Investigations on prolyl hydroxylase activity show that the levels of the enzyme are higher in younger subjects than in older ones.

Injury and Inflammation: Collagen synthesis occurs in the second phase of wound healing (14). During the early part of the first phase, fibroblasts migrate and proliferate in response to injury. There is a significant increase in collagen synthesis by the newly arrived

fibroblasts. Because collagen modulates normal cells behavior, including migration, proliferation and specific gene expression and differentiation, it plays a major role in wound healing. Collagen is also the principal determinant of tissue bio-mechanical properties. The level of prolyl hydroxylase reflects the collagen synthetic activity of tissues. In wound healing collagen synthesis and prolyl hydroxylase are markedly elevated. Injury may be regarded as one of the stimuli for prolyl hydroxylase activity. In adult tissues, inflammation usually accompanies injury. It contributes the stimulus for tissue repair (15). Superoxide is a major chemical mediator of inflammation and is produced by the triggered neutrophils and macrophages in inflammed tissues (16). Previous studies from this laboratory have shown that superoxide is an important signal for stimulating collagen synthesis (17).

Tissue Oxygenation: Superoxide is a product of the one electron reduction of molecular oxygen. It is the first step in the metabolic fixation of oxygen and is present in all cells (18). Increased tissue levels of oxygen and oxidants, e.g. ozone, give rise to increased levels of superoxide. In the first step of oxygen metabolism, oxygen molecules readily accept single electrons to form the highly reactive free-radical superoxide (Fig. 1.2). Once superoxide is formed, it can undergo reduction to form other active oxygen species such as: hydrogen peroxide (H_2O_2) and the very reactive hydroxyl free radical (OH^-) which can react with DNA and cause single strand breaks. Many lines of evidence indicate that active oxygen may be involved in inducing a pro-oxidant state which modulates gene

expression and increases cellular metabolic activity (19). Superoxide is also a mutagenic agent (20). It can cause single strand breaks in DNA, chromosomal damage, and sequence rearrangements in normal cells in culture (21,22). In addition to these structural genetic changes, superoxide may participate in epigenetic mechanisms that cause alteration of gene expression (23). Superoxide is also cytotoxic to living cells and organisms. Superoxide dismutase (SOD) is the only enzyme which can protect against damage caused by this free radical (24).

SOD activity in cells plays an important role in cell survival (25) which means, within a given organism, raising the level of SOD should provide increased tolerance for superoxide damage. SOD levels also increase during development and aging (26). Superoxide itself appears to be a stimulus for collagen synthesis (18) and the inhibition of intracellular SOD results in a marked stimulation of collagen synthesis and increase of prolyl hydroxylase activity. Therefore, SOD may help in regulation of prolyl hydroxylase activity (4). Exposure of cells and tissues to oxidant toxins: eg, ozone (27) etc increases prolyl hyroxylase activity. It is also clear from our previous studies that if cells and tissues are presented with certain superoxide-generating drugs including Bleomycin (6,28) and paraquat (17,25,29), the elevated cellular level of prolyl hydroxylase activity and collegen synthesis is accompanied by increased activity of PADPRS (30). These agents are known to act through oxygen-free radicals.

$$[1] \quad O_2 + e^- \rightarrow O_2^-$$

$$[2] \quad 2H^+ + 2O_2^- \rightarrow H_2O_2 + O_2$$

$$[3] \quad O_2^- + H_2O_2 \rightarrow \cdot OH + OH^- + O_2$$

$$[4] \quad \cdot OH + DNA \rightarrow DNA\cdot + H_2O$$

One electron reduction of oxygen gives rise to superoxide. Reactions of superoxide generate other free radicals.

$$2O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

Superoxide dismutase converts superoxide to hydrogen peroxide.

Fig. 1.2: Formation and Reactions of Superoxide and other Oxygen Radicals.

Ascorbate: Ascorbate is an essential co-factor in the hydroxylation of proline and lysine to form hydroxyproline and hydroxylysine, amino acids critical to the function of collagen. Ascorbate is regarded as a fundamental signal for collagen production. It also stimulates collagen synthesis at the transcriptional or mRNA processing level (31). This latter function in collagen synthesis is different from its function as a cofactor in the post-translational hydroxylation of collagen. Ascorbate serves as an important source of superoxide by supplying the reducing electrons in the presence of transition metal ions (32,33) (Fig. 1.3).

All of these unrelated conditions which regulate the cellular level of prolyl hydroxylase and collagen synthesis have only one common feature, that is their ability to generate superoxide. Superoxide may act as a biological signal for stimulating connective tissue proliferation.

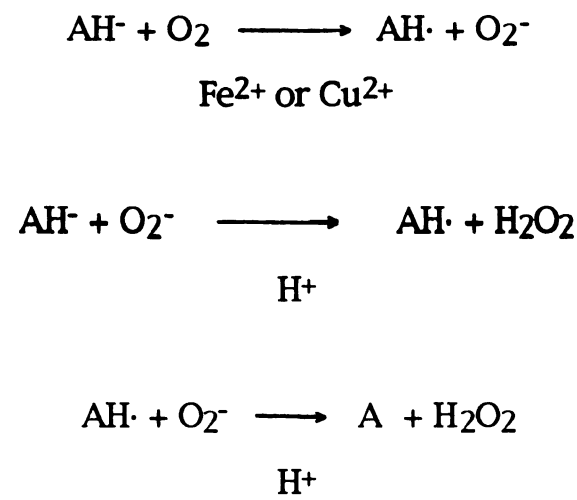


Fig. 1.3: Generation of Superoxide and other Oxygen Radicals by Ascorbate. AH^- = Ascorbate; AH· = acorbate free radical, A = dehydroascorbate. The first reaction is the rate determining step. Other reactive species including OH- and OH· are formed via the Haber-Weiss reaction from H_2O_2 and ascorbate (20).

III. Poly ADP-ribose Synthetase (PADPRS):

General Concept: Poly (ADP-ribose) synthetase [also called poly ADP-ribose polymerse or NAD^+: poly (adenosine diphosphate D-ribose) ADP-D-ribosyl-transferase] is a chromatin-bound enzyme and is localized in the nucleus in most eucaryotic cells (34,35). It catalyzes the successive transfer of ADP-ribose moieties from substrate NAD^+ to nuclear proteins such as histones and to the enzyme itself. The reaction is shown in Fig. 1.4:

This post-translational modification reaction is involved in various chromatin functions by modulating the interactions between structural proteins and DNA. This enzyme activity is stimulated by DNA strand breaks (36). The polymer may alter the structure and function of chromosomal proteins to facilitate the DNA repair process. The PADPR of nuclear protein is shown on Fig. 1.5.

The Effect of Active Oxygen on PADPRS Activity: Active oxygen and other oxidants usually act as part of the mechanism of inflammation and tumor promotion (37). The mechanism of action of tumor promotors and oxidants appears to involve the modulation of the expression of growth- and differentiation-related genes and cytotoxic effects (38). Active oxygen and poly ADP-ribose affect the redox state and energy metabolism of the cell, and active oxygen induces DNA single-strand breaks that strongly activate PADPRS activity. Therefore, active-oxygen induced PADPR of chromosomal protein is likely to play a role in oxidant promotion (39).

As discussed above, the factors that can cause increase in collagen synthesis and increase in superoxide generation can affect cellular levels of poly ADP-ribosylation. Ascorbate and phorbol ester (PMA) are such agents (40,41).

The Effects of Aging on ADP-ribosylation: The level of PADPRS is elevated in tissues undergoing development and differentiation (42,43). The effect of age on PADPR-mediated processes appears to involve the state of nuclear protein substrates for PADPRS. In the young organism, high rates of poly ADP-ribosylation of certain histones (High Mobility Group, HMG) is observed (35,44), whereas age-related condensation of HMGs in older organisms results in decreased PADPR (45,46). This alteration causing decrease in the substrate activity of nuclear protein may explain the effect of age on many regulatory processes including the modulation of collagen synthesis as a function of age.

PADPRS Inhibitors: Inhibition of PADPRS has been used effectively to explain the involvement of PADPR in many normal and pathobiological processes. In the past few years, a number of inhibitors of PADPRS activity have been described (Table 1.1) (47). We have used some of these inhibitors to establish a role for PADPRS in the regulation of prolyl hydroxylase.

PADPRS

$nNAD^+ + acceptor \longrightarrow (ADP\text{-}Ribose)n\text{-}acceptor + nNicotinamide + nH^+$

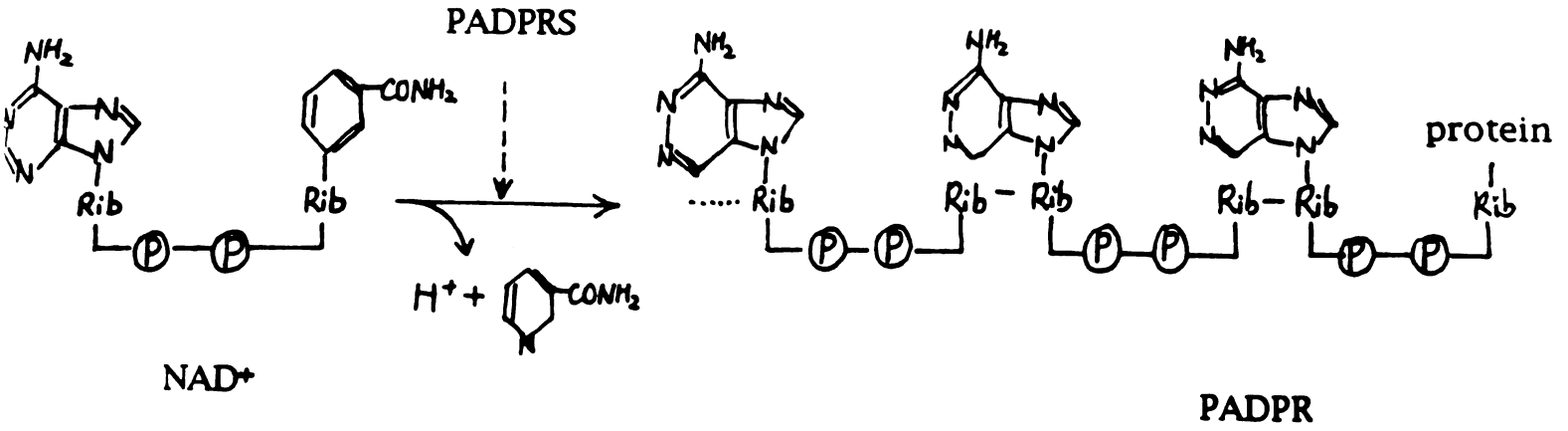


Fig. 1.4 Poly ADP-ribosylation

Proposed Mechanism for the Regulation of Gene Expression by PolyADP-ribosylation

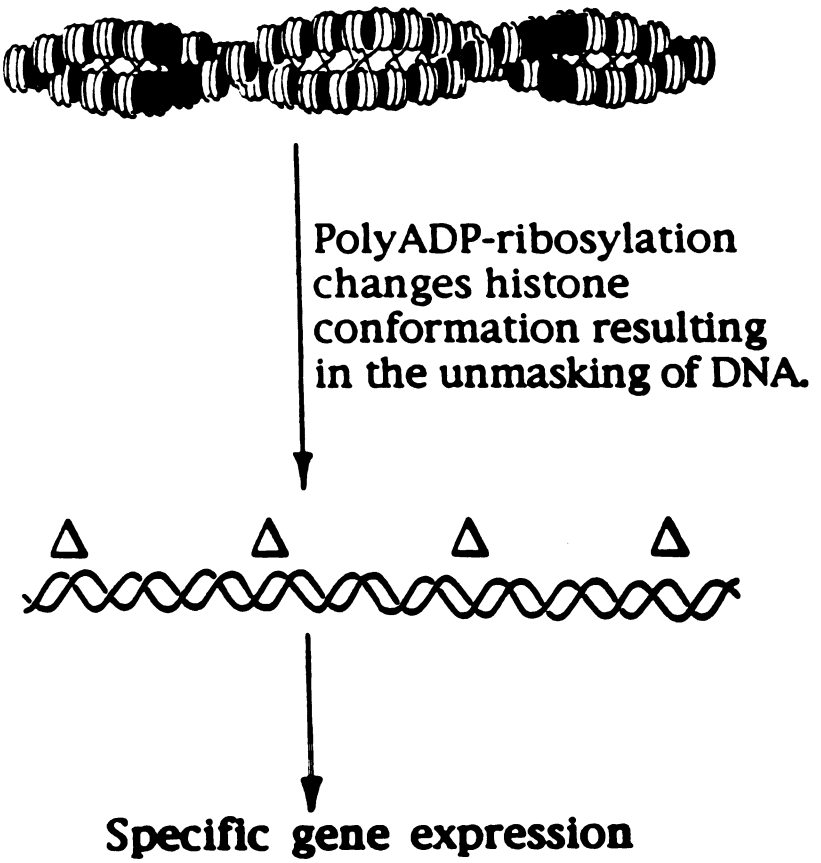


Fig. 1.5

Compounds	Concentration	Inhibition(%) at:
3-Aminobenzamide	2.0 mM	60
6-Aminonicotinamide	1.5 mM	60
	2.0 mM	58
Theophylline	1.0 mM	59
Benzamide	1.0 mM	56
2-acetyl pyridine	1.0 mM	63
Thymidine	1.0 mM	56

Table 1.1: Inhibitors of PADPRS. The inhibition data are taken from ref. 84.

PADPRS inhibitors are usually substrate analogues. These compounds inhibit the synthesis of PADPR and prevent the decrease in cellular NAD content in intact cells (48,49). Among these inhibitors, 3-aminobenzamide (3AB) has particularly useful properties since it is a competitive inhibitor of the enzyme with a relatively low Ki, very little cytotoxic effects and probably little metabolic involvement in other biochemical reactions (50-52). It has been shown that 2.5 mM 3AB, 5.0 mM nicotinamide, and 1.0 mM theophylline completely block cellular poly(ADP-ribose) synthetase activity in $P388D_1$ cells and in human lymphocytes (53). 1.0 mM nicotinamide and 1.0 mM 3AB caused complete and specific inhibition of endogenous PADPRS in L1210 cells (54). Our studies suggested that PADPRS inhibitors block the induced increase in prolyl hydroxylase activity, suggesting regulation by PADPR.

IV. Involvement of G-protein and Protein Kinase C Signalling Pathways in the Control of Gene Expression.

General Concept: G-proteins (so called guanine nucleotide-binding protein) are involved in membrane-bound transduction of chemically and physically coded information (55). The function of G-proteins in the membrane signalling pathway is to couple with receptors to their respective effector proteins (56). The most important members in G-protein family are: a stimulatory G-protein (Gs) and an inhibitory G-protein (Gi). These G-proteins play major role in hormone-mediated stimulation and inhibition of adenyl cyclase

activity (57). G-proteins have three subunits: α, β, and γ. The α-subunit of G-protein is a substrate for ADP-ribosylation catalyzed by bacterial toxins, like cholera toxin (58) and pertussis toxin (59).

G-proteins Serve As Substrates for ADP-ribosylation Catalyzed by Cholera Toxin and Pertussis Toxin: Although both of these bacterial toxins act as specific ADP-ribosylating enzymes for G-protein, they function at different effector sites of G-protein. The α-subunit of stimulatory G-protein (Gsα) can be ADP-ribosylated by cholera toxin, while the α-subunit of inhibitory G-protein (Giα) is substrates for ADP-ribosylation by pertussis toxin (60). The ADP-ribosylation of Giα activated by pertussis toxin causes an impaired ability to interact with its receptor. In contrast, when Gsα is activated by cholera toxin, this results in stimulated G-protein transduction mediated by its receptors. Cholera toxin promotes the expression of certain genes (61,62). This has been shown to be due to the involvement of G-protein (63). The inhibition of the stimulatory effect of cholera toxin on certain biochemical events by pertussis toxin is taken as evidence supporting the involvement of G-proteins (64-66). Because of the observation that the effects of cholera toxin can be blocked by inhibitors of PADPRS, it has been suggested that cholera toxin may also induce nuclear PADPR-mediated pathways (67). I have examined the effect of cholera toxin and pertussis toxin on prolyl hydroxylase to determine if G-protein and signalling pathway are involved in the modulation of prolyl hydroxylase.

Protein Kinase C: An important component of the G-protein signalling pathway is the membrane bound enzyme protein kinase C (PKC). PKC is activated by diacylglycerol and it is Ca^{2+}-dependent. PKC is thought to phosphorylate and thereby activate plasma membrane proteins, resulting in the stimulation of the signal for cell proliferation or inhibition of certain cellular processes depending on the cell type and the duration of PKC activation (68). In some cells the activation of PKC increases the transcription of specific genes. The promotors of at least some of these genes contain a common transcriptional enhancer sequence that is recognized by a gene regulatory protein whose activity is stimulated by PKC activation (69). The stimulation of PKC activity resulted in the phosphorylation of a 40-kilodalton (kDa) protein which was related to the α-subunit of a G-protein in platelets (70). PKC activity is modulated by the tumor promotor PMA which is also a superoxide generator (71.72). PKC acts as a recepor for PMA and is directly activated by it (73). The effect of the PMA to increase PADPRS has been well documented (74). Our studies showed elevated activity of prolyl hydroxylase in the presnece of PMA. Thus, in order to examine if PKC mediated mechanisms may play a role in the modulation of prolyl hydroxylase, I examined the effect of inhibiting PKC on the increase in prolyl hydroxylase activity induced by PMA and cholera toxin and also by ascorbate. My observations suggest the participation of PKC mediated signalling pathways in the modulation of prolyl hydroxylase. My hypothesis on the regulation of prolyl hydroxylase by mechanisms involving PADPR, PKC and G-protein mediated signalling pathways is shown in Fig. 1.6. In my hypothesis, PADPRS plays a central role in

the regulation of prolyl hydroxylase. PADPRS is known to regulate gene expression. Several agents and conditions that are known to increase collagen synthesis and prolyl hydroxylase activity are accompanied by increased levels of active oxygen species including superoxide. The stimulatory effect of some of these on PADPRS is already well known. Both PMA and cholera toxin are known to cause increased levels of superoxide. These agents therefore also increase PADPRS and this has been documented.

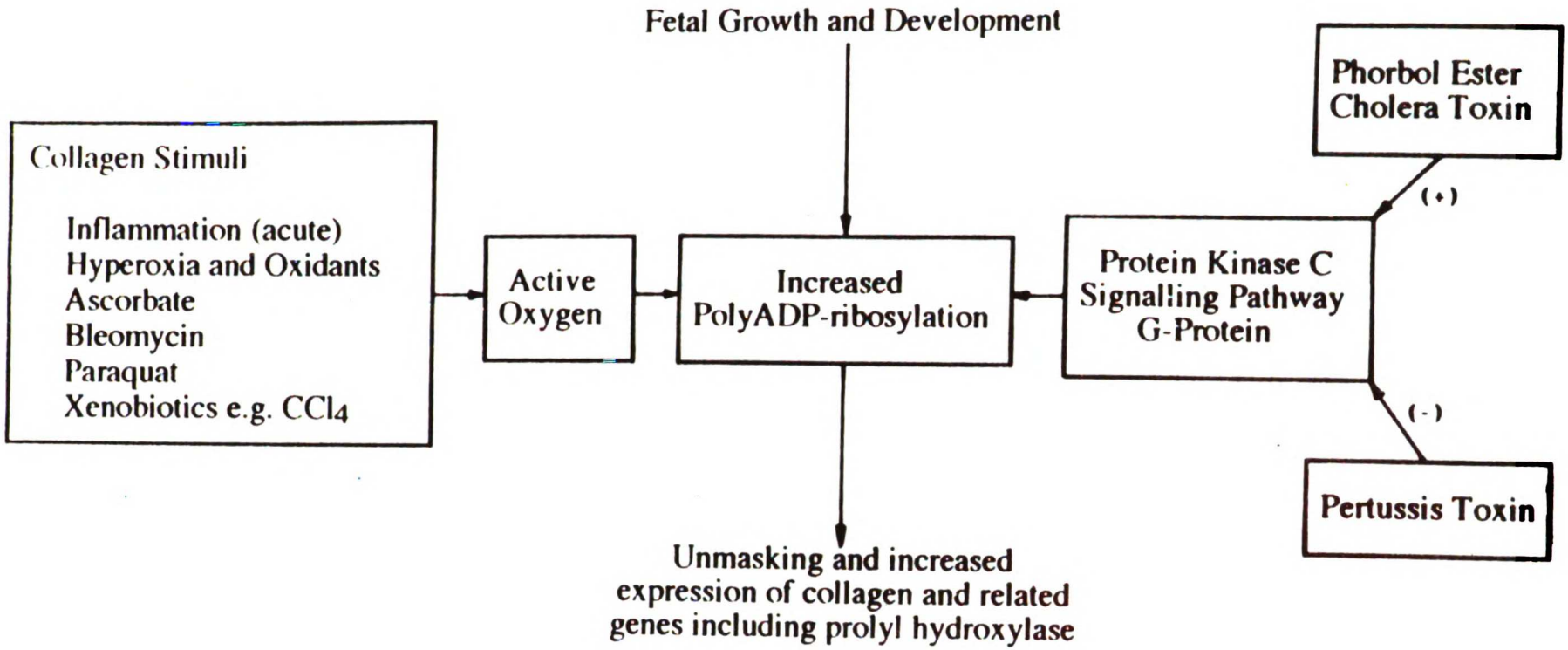

Fetal Growth and Development
Phorbol Ester
Cholera Toxin
Collagen Stimuli
Inflammation (acute)
Hyperoxia and Oxidants
Ascorbate
Bleomycin
Paraquat
Xenobiotics e.g. CCl_4
Active
Oxygen
Increased
PolyADP-ribosylation
Protein Kinase C
Signalling Pathway
G-Protein
(+)
(-)
Pertussis Toxin
Unmasking and increased
expression of collagen and related
genes including prolyl hydroxylase

Fig. 1.6

Chapter 2. Materials and Method

I. Materials

All chemicals used in these studies were obtained from Sigma Chemical Co. Radiolabeled proline, 3,4-[$^{3}H_2$]-L-proline (30 Ci per mmole) or [^{14}C-U]-L- proline (250 mCi per mmole) were purchased from New England Nuclear. Tissue culture media and other supplies were obtained from the UCSF Tissue culture Facility. IMR-90 cells in the eighth passage were purchased from the Institute for Medical Research, Camden, NJ. Primary cultures of human fetal fibroblasts were prepared from tissues obtained from therapeutic abortion materials. Signed consent for the use of this tissue for research purpose was obtained under an approved human subjects research protocal at UCSF. Dermal fibroblasts from these clinical specimens were used between the fourth and eighth passages. The reason for using early passage cells is that long term cells change their biological behavior and the cellular levels of PADPRS are markedly altered (75,76). This results in alteration in cell proliferation and differentiation, and changes in specific gene expression.

II. Cell Cultures

Cells were seeded in 60 mm plastic culture dishes or T-75 flasks at a density of $5x10^3$ cells/cm^2 in Eagles minimal essential mediun containing 10% fetal calf serum. The cultures reached confluence by the fifth and sixth day for fetal and neo-natal cells. All experiments were performed with confluent cultures.

All additives were dissolved in the medium before addition. Ascorbate solutions were always prepared fresh, just prior to addition to the cultures. Preliminary experiments in our laboratory showed that ascorbate is consumed rapidly in the culture medium in contact with cells, and little ascorbate can be detected 3-4 hours after addition.

After incubation for the indicated periods, the cells were rinsed once with chilled Ca^{2+} and Mg^{2+} - free phosphate buffered saline solution. All subsequent operations were carried out at 4^o. The cells were homogenized in the following buffer: (10.0 mM Tris HCl, 100 mM NaCl, 100 mM glycine, 0.01 mM dithiothreitol, and 0.1% Triton X-100, pH 7.4). The homogenates were kept in a -80^o freezer until used in enzyme assays.

III. Assay for Protein

Protein concentration of homogenates was measured by using Coomassie Brilliant Blue (77) with a commercially available assay kit (BioRad Protein Assay).

IV. Assay for Prolyl Hydroxylase Activity

The procedure for prolyl hydroxylase assay was essentially as same as described in our previous studies (17). The assay originally described by Hutton *et al.* (78) was used with minor modifications. This method is based on the release of tritium from ^{3}[H]-proline-labeled unhydroxylated collagen from 11-day-old chick embryo cartilage culture used as the substrate (Fig. 2.1).

Preparation of Labeled Substrate: Unhydroxylated ^{3}H-Proline labeled substrate was prepared by short term organ culture of 9- to 11-day-old chicken embryo tibiae in Dulbecco modified Eagle's minimum essential medium buffered at pH 7.4 with 25 mM HEPES. The intracellular prolyl hydroxylase was inhibited by the addition of 1.0 mM 2,2'-dipyridyl in the presence of 3,4-[$^{3}H_2$]-L-proline. Incubation was carried out at 37^{o} for 6-8 h. The labeled tibiae were homogenized, and the homogenate was dialyzed against H_2O until no radioactivity could be detected in the dialyzate. The retentate was centrifuged at 15,000 x g. The substrate was present in the soluble fraction, in the supernatant.

Prolyl Hydroxylase Reaction: The reaction mixture contained 100 mM Tris HCl (pH7.6), 1.0 mM ferrous ammonium sulfate, 200,000 cpm of [^{3}H] proline-labeled unhydroxylated collagen, 0.5 mM ascorbate and cell homogenate (0.5 to 1.0 mg protein) in a final volume of 2.0 ml. The reaction was started by the addition of ascorbate, incubation was carried out at at 37° for 10 min. The reaction was stopped by adding 0.2 ml 50% trichloroacetic acid and tritiated water was separated by vaccum distillation. 1.0 ml of the distillate was mixed with 15 ml of Aquasol and counted for radioactivity in a Parkard Tri-Carb Scintillation Spectrometer. The activity of prolyl hydroxylase was expressed as d.p.m. ^3H_2O released per mg of protein in the cell homogenate.

$$\text{-(}^3\text{H-Pro-Gly)-} + \alpha\text{-ketoglutarate} + O_2 \xrightarrow[\text{Ascorbate}]{Fe^{2+}} \text{-(Hyp-Gly)-} + \text{Succinate} + CO_2 (\uparrow) + {}^3\text{H}$$

$$2\,[^3H^+] + H_2O \rightleftharpoons {}^3H_2O + 2\,[^1H^+]$$

^3H_2O is collected by vaccum distillation and the radiactivity is assayed as a function of enzyme activity.

Fig. 2.1: Prolyl hydroxylase reaction

V. Determination of PADPRS Activity

Poly ADP-ribose synthetase activity was measured by a modification of the method of Ghani and Hollenberg (52,79). The method is based on the incorporation of ^{3}H-adenine-NAD$^+$ into acid-insoluble material in permeabilized cells. The specific substrate of PADPRS is NAD$^+$, to which intact cell membranes are impermeable. Isolated nuclei are unsuitable because the process of nuclear isolation induces DNA strand breaks and activates PADPRS activity (39). Fibroblasts were grown in 60 mm culture dishes to confluence and treated as described. The treated cells were washed three times with ice-cold PBS (Mg^{2+}, Ca^{2+} free) solution. The neo-natal dermal fibroblast were made permeable to ^{3}H-NAD by a mild, brief hypotonic treatment by a modification of a published method (80). The plates were flooded with 1.0 ml of a hypotonic solution containing 9.0 mM HEPES, pH 7.8, 5.0 mM dithiothreitol (DTT), 4.5% (w/v) dextran (average molecular weight 110,000), 1.0 mM EGTA and 4.5 mM $MgCl_2$. The plates were kept at 4^o, until more than 90% of the cells had become permeable to Trypan blue. This was routinely achieved after 60 min. After permeabilization, the plates were transferred to a water bath at 25^o and the ADP-ribosyl synthetase assay was started by the addition of 200 µl of a solution containing 200 mM HEPES pH 7.8, 750 mM KCl, 5.0 mM DTT, 4.5% (w/v) dextran,1.35 M sucrose, 7.0 mM EGTA, 4.5 mM $MgCl_2$, 6.0 mM isonicotinic acid hydrazide, 18.0 mM NaF and 600 µM ^{3}H-NAD (20mCi/mM). The enzyme incubation was continued for 10 min and then terminated by the addition of 2 ml of ice-cold 20% (w/v) trichloroacetic acid (TCA).

The precipitate was collected on a glass fiber filter with a pore size of 0.45 microns and washed successively with 10 ml 20% TCA, 10 ml 10% TCA and finally with 10 ml 5% TCA. The precipitate was then washed once with 10 ml 95% ethanol to remove the TCA. The filter and the precipitate were dissolved in BTS-45° tissue solubilizer (Beckman). The radioactivity incorporated in TCA insoluble fractions was used as a measure of PADPRS activity.

Chapter 3. Characterization of Model for Studying Prolyl Hydroxylase Activity in Cell Cultures.

Fibroblasts have been used extensively in studies an collagen synthesis and prolyl hydroxylase. I used neonatal human dermal fibroblasts in these studies, except where fibroblasts of various ages were compared. It was necessary to determine if the conditions used in thses experiments were inhibitory to fibroblasts. I examined the toxicity of each agent which was presented to my cell cultures. In the past few years, many inhibitors of PADPRS activity have been described (47). Among these inhibitors, 3-aminobenzamide (3AB) and 6-aminonicotinamide (6AN) have been used commonly, since they are competitive inhibitors of the enzyme with very little cytotoxic effects and probably little metabolic involvement in other biochemical reactions (51-53). In order to determine if these agents were cytotoxic, dermal fibroblasts were treated with ascorbate and PADPRS inhibitors added singly or together for 1 h at 37° (in 5% CO_2-air) and washed with (Mg^{2+} and Ca^{2+} free) PBS buffer once. The number of dead cells was examined by Trypan-blue exclusion method (81). The results (Table 3.1) demonstrated that the agents were not cytotoxic under the conditions used in my experiments.

Experiment	Total cells (cells/ml)x10^4	#(dead cells) (cell/ml)x10^4	% Dead cells
Control	3.8	0.6	15.7
+Ascorbate, 0.35 mM	4.1	0.5	12.2
+3AB, 2.0 mM	3.5	0.5	14.3
+Ascorbate, 0.35 mM +3AB, 2.0 mM	2.9	0.3	10.3
+6AN , 2.0 mM	3.6	0.6	16.7
+Ascorbate, 0.35 mM +6AN , 2.0 mM	3.8	0.4	10.5

Table 3.1: Lack of cytotoxity of experimental conditions used. No additions were made in the control. There was no significant difference in the number of dead cells in various treatments.

In order to examine if the addition of various agents affected cell growth, we cultured human dermal fibroblasts for 8 days with or without different agents. Fibroblasts were plated at a density of 25,000 cells per 60 mm culture dish. The proliferation of fibroblasts was monitored by assaying for the protein content in the cell layer, as a function of culture time. These results (Fig. 3.1) showed that the growth patterns of the cells treated with various agents were identical. The reagents did not affect cell proliferation.

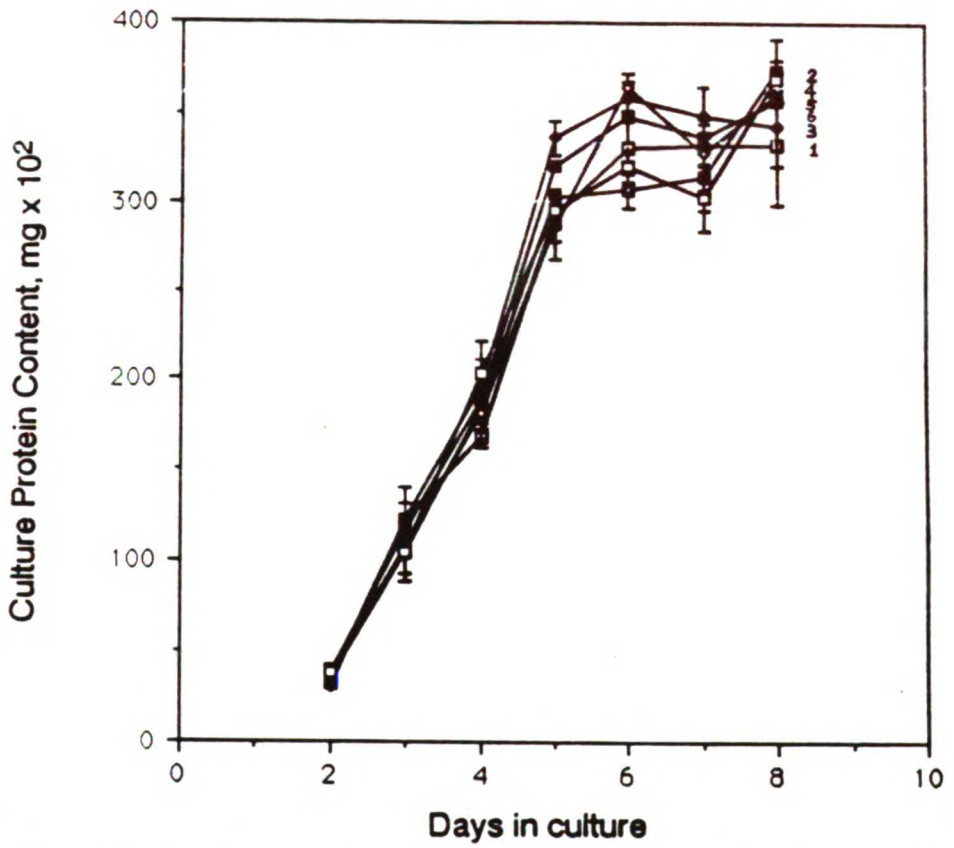


Fig. 3.1: The effect of various additives on the rate of cell proliferation. Curve 1, control; curve 2, cells treated with 0.35 mM ascorbate; curve 3, cells treated with 2.0 mM 3AB; curve 4, cells proliferation in the presence of 2.0 mM 6AN; curve 5, cells treated with 0.35 mM ascorbate and 2.0 mM 3AB and curve 6, cells exposed to 0.35 mM ascorbate and 2.0 mM 6AN. There was no significant effect of addition of the various reagents on the growth rate of cells.

Chapter 4. Effect of Ascorbate on Prolyl Hydroxylase Activity

Hydroxylation of collagen proline is a very important step in the collagen metabolism. Although it is well known that ascorbate plays a major role in this reaction, the mechanism of its action remains poorly understood. Two sites of ascorbate action on collagen synthesis have been recognized. First, ascorbate is an essential cofactor for collagen hydroxylating enzymes and it regulates the post-translational modification of collagen lysyl and prolyl residues which are essential for efficient procollagen triple helix formation. Second, it stimulates collagen synthesis at a transcriptional, or mRNA processing level. Ascorbate stimulates normal collagen prolyl hydroxylation by cultured fibroblasts. Previous studies showed that collagen synthesis in fibroblast culture can be increased by the addition of ascorbate to the culture medium (82,83). In my studies, I needed to characterize the culture system to determine the optimal conditions for examining the effect of ascorbate on prolyl hydroxylase. In order to determine the best concentration of ascorbate for my experiments, I examined the effect of various concentrations of ascorbate on the activity of prolyl hydroxylase. Confluent cultures were exposed to different concentrations of ascorbate, and prolyl hydroxylase activity was determined after 24 hours. The results of these experiments are presented in Fig 4.1. As seen in this experiment, the previously reported ascorbate concentration of 0.35 mM caused maximal increase in the prolyl

hydroxylase activity. Concentrations higher than this caused a decrease. The reason for this decrease was not examined.

It was also of interest to determine the effect of the length of exposure of fibroblasts to ascorbate on the levels of prolyl hydroxylase. Ascorbate is rapidly oxidized in the culture medium and little ascorbate remains in the medium after 30 min (23). I examined the activity of prolyl hydroxylase at various times after exposure to 0.35 mM ascorbate. The data from this experiment are presented in Fig 4.2. The enzyme activity showed significant increase within 15 min and the activity continued to increase up to 24 hours, although the rate of increase was slower after 6-12 hours following ascorbate exposure.

Based on the results of this experiment, all subsequent experiments were carried out with 24 hours exposure of confluent cultures to 0.35 mM ascorbate.

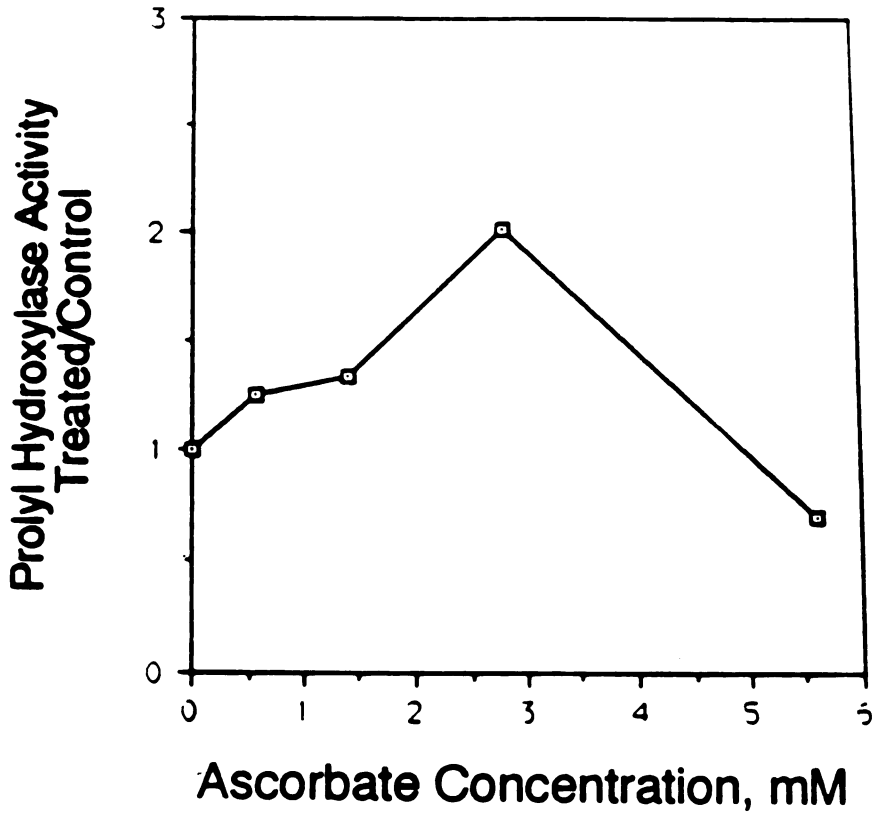


Fig. 4.1 Effect of Ascorbate Concentration on Prolyl Hydroxylase Activity. The highest enzyme activity was seen in the presence of 0.35 mM ascorbate.

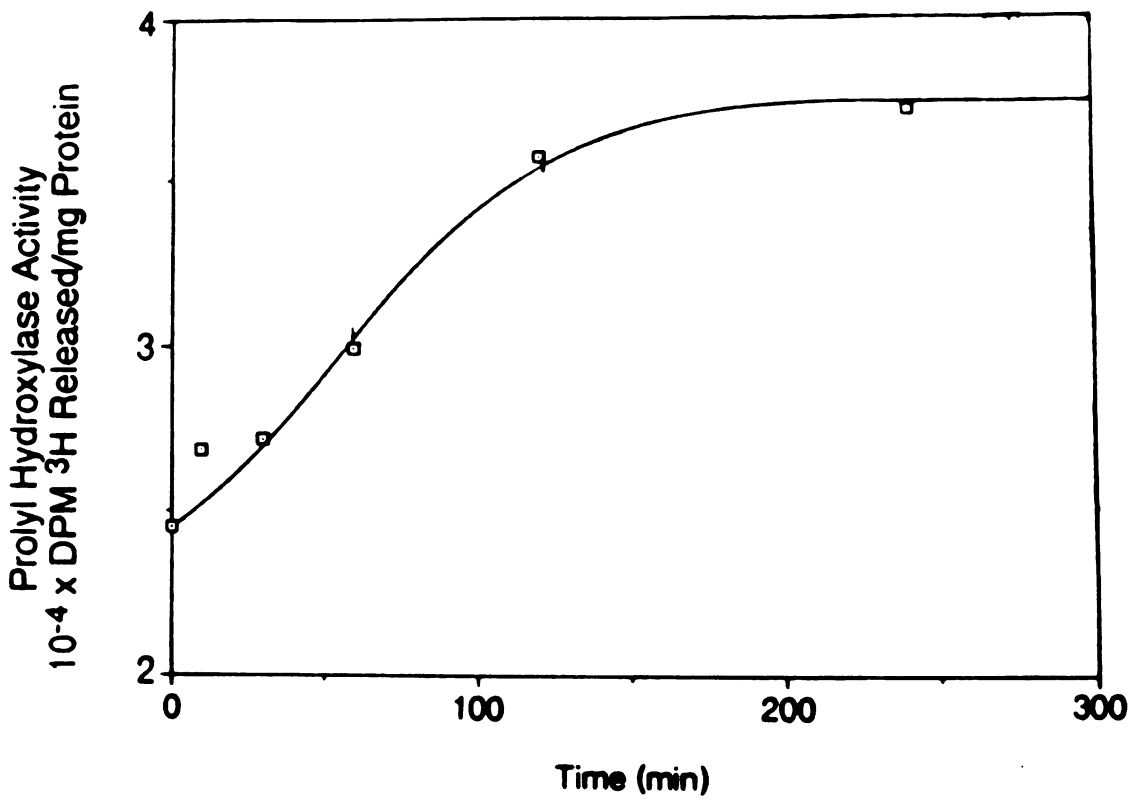


Fig. 4.2 Time Couse of Increase in Prolyl Hydroxylase after Exposure to Ascorbate. Prolyl hydroxylase activity was increased by 50% within 60 min after exposure to 0.35 mM ascorbate. The enzyme activity continued to increase until 24 hours.

Chapter 5. Involvement of PADPRS in the Modulation of Prolyl Hydroxylase Activity.

Effect of Ascorbate on PADPRS: Our previous studies showed that the fibrogenic agents ozone (27) and Bleomycin (6,28) increased collagen synthesis, prolyl hydroxylase activity, and these compounds also markedly elevated PADPRS activity. If PADPRS is a part of the regulatory pathway for collagen and prolyl hydroxylase, its activity should be modulated by other agents that increase collagen synthesis. This is also predicted by the observation that ascorbate generates free radicals which increase PADPRS (23,31). The effect of ascorbate on PADPRS has not been reported previously. Since ascorbate increases collagen synthesis and prolyl hydroxylase activity in fibroblast cultures (17,18), it was of interest to determine if it also increased PADPRS activity and if there was a correlation betwen these enzymes. I examined the effect of exposure of fibroblasts to 0.35 mM ascorbate on PADPRS levels. As seen in Fig. 5.1, ascorbate caused a rapid increase in PADPRS activity. The enzyme activity nearly doubled after 2 h of exposure to ascorbate. These data are the first to show that ascorbate induces PADPRS. This observation also supported my hypothesis that PADPRS may play a role in the modulation of prolyl hydroxylase. This question was further examined by inhibiting PADPRS in the presence of ascorbate. My rationale in this experiment was that if increased PADPRS activity is involved in the modulation of prolyl hydroxylase, lowering PADPRS activity by inhibitors should abolish the stimulatory effect of increasing PADPRS.

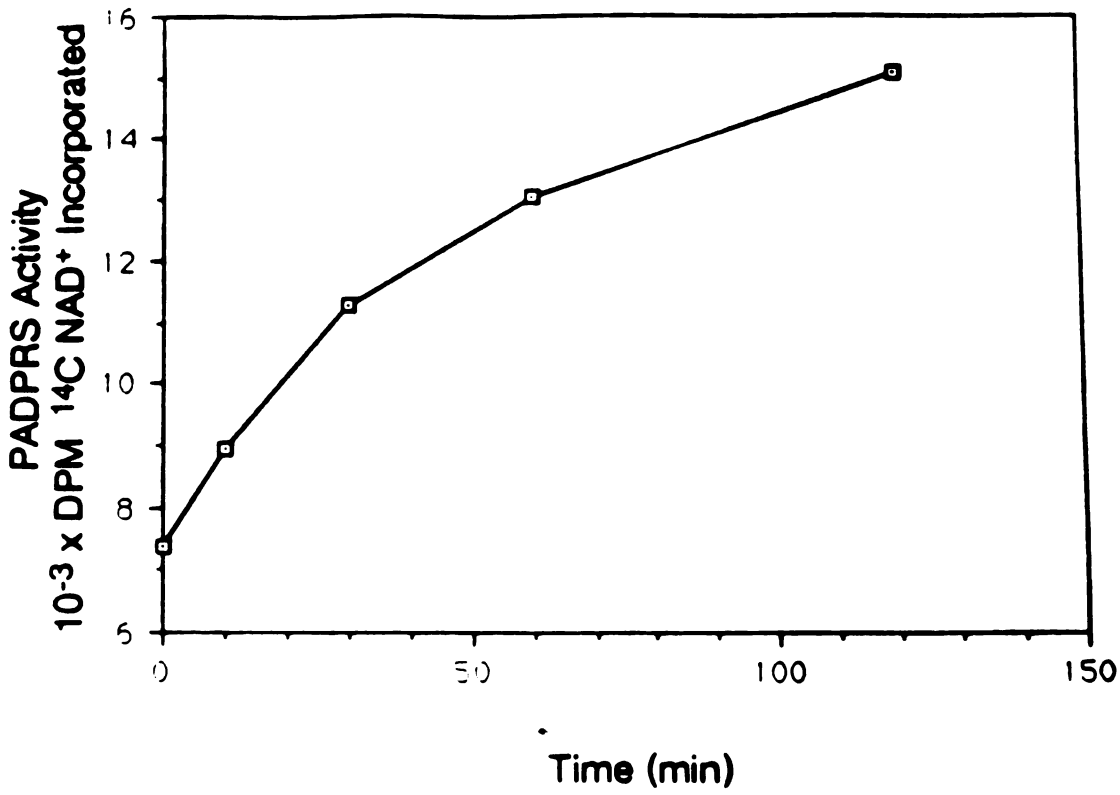


Fig. 5.1 0.35 mM Ascorbate caused a rapid increase in PADPRS activity during 2 h exposure.

Effect of PADPRS Inhibitors on the Ascorbate Mediated Increase in Prolyl Hydroxylase: If PADPRS plays a role in the modulation of prolyl hydroxylase, then inhibitors of PADPRS should have an attenuating effect on the increase in prolyl hydroxylase by ascorbate. I examined the effect of PADPRS inhibitors on prolyl hydroxylase activity in the presence of ascorbate, in several different types of fibroblast cultures. In preliminary experiments, I examined the possibility that these inhibitors may directly inhibit prolyl hydroxylase. I found that the inhibitors had no effect on the *in vitro* reaction of purified chicken embryo prolyl hydroxylase. Thus, any changes in cellular prolyl hydroxylase would result from a decrease in the enzyme activity caused at some other level, presumably at the level of enzyme expression as stated in my hypothesis.

As seen in Table 5.1, ascorbate increased the activity of prolyl hydroxylase in all cell types examined. The ascorbate-induced increase in prolyl hydroxylase was comparable in the "young" cells, namely 22 w fetal dermal cells, IMR-90 cells and in neo-natal dermal fibroblasts. In each case the enzyme activity exibited nearly a two-fold increase after exposure to ascorbate. In contrast, the fibroblasts derived from the skin of a 78 y old subject showed only a 21 % increase.

The ascorbate induced increase in prolyl hdroxylase was abolished by inhibitors of PADPRS. In case of the 22 w fetal fibroblasts, IMR-90 fibroblasts and 78 y dermal fibroblasts, only two inhibitors, 3AB

and 6AN were used. These agents effectively abolished the increase in prolyl hydroxylase in all cells.

The involvement of PADPRS was further confirmed by the use of several other inhibitors of this enzyme. Neo-natal dermal fibroblasts were exposed to ascorbate or ascorbate and one of the following inhibitors, 3AB, 6AN, theophylline, benzamide, 2-acetyl pyridine or thymidine. The concentrations of these inhibitors are indicated in the legend to Table 5.1. In all cases, the inhibitors blocked the ascorbate induced increase in prolyl hydroxylase. These results demonstrate that PADPRS is a site for the modulation of prolyl hydroxylase activity.

A comparision of the data in Fig.s 4.1 and 5.1 shows that both PADPRS and prolyl hydroxylase activities are elevated in cells exposed to ascorbate. The increase in PADPRS is more rapid than the increase in prolyl hydroxylase. The lag in the induction of increased prolyl hydroxylase can be explained by the time needed for the activation of PADPRS mediated pathways for gene expression.

Table 5.1; Inhibitors of PADPRS abolish the ascorbate-induced increase in prolyl hydroxylase activity

Cell type and experiment	Enzyme activity dpm ^3H/mg protein x 10-4	Treated/Control	Inhibitor/ascorbate
Fetal dermal fibroblasts (22 weeks)			
Control	3.14 ± 0.08		
+Ascorbate	6.00 ± 0.18	1.19	
+Ascorbate + 3AB	3.60 ± 0.14	1.15	0.60
+Ascorbate + 6AN	2.97 ± 0.06	0.96	0.50
IMR-90 fibroblasts			
Control	1.68 ± 0.09		
+Ascorbate	3.44 ± 0.09	2.05	
+Ascorbate + 3AB	1.76± 0.23	1.05	0.51
+Ascorbate + 6AN	1.64 ± 0.26	0.98	0.48
Adult dermal fibroblasts (78 years)			
Control	2.83 ± 0.16		
+Ascorbate	3.43 ± 0.23	1.21	
+Ascorbate + 3AB	3.13 ± 0.16	1.11	0.91
+Ascorbate + 6AN	2.72 ± 0.14	0.96	0.80

Neonatal dermal fibroblasts (foreskin)			
Control	3.08 ± 0.26		
+Ascorbate	5.59 ± 0.45	1.18	
+Ascorbate + 3AB	3.14 ± 0.07	1.11	0.61
+Ascorbate + 6AN	3.22 ± 0.13	1.05	0.58
+Ascorbate + Theophylline	3.31 ± 0.32	1.07	0.59
+Ascorbate + Benzamide	3.15 ± 0.19	1.02	0.56
+Ascorbate + 2-acetyl pyridine	3.54 ± 0.38	1.15	0.63
+Ascorbate + Thymidine	3.12 ± 0.37	1.01	0.56

All cells were used between 5th and 8th passages. No additions were made in the control. In experiment to examine the stimulatory effect of ascorbate, 0.35 mM ascorbate was added. In order to examine the effect of PADPRS inhibitors on the stimulatory effect of ascorbate, the cultures received in addition to 0.35 mM ascorbate, one of the following, 2.0 mM 3-aminobenzamide (3AB), 2.0 mM 6-aminonicotinamide (6AN), 1.0 mM theophylline, 1.0 mM benzamide, 1.0 mM 2-acetyl pyridine, 1.0 mM thymidine. The cells were harvested 24 h after the addition of ascorbate and/or inhibitors of PADPRS. Prolyl hydroxylase activity was determined as described elsewhere.

Chapter 6. Involvement of PADPRS in the Modulation of Prolyl Hydroxylase as a Function of Cell Proliferation.

Previous studies have shown that collagen synthesis and prolyl hydroxylase activity are modulated as a function of cell proliferation in culture (82). In earlier studies it was shown that collagen synthesis is maximal in confluent cultures, and that the levels of prolyl hydroxylase lag behind the growth curve, with highest activity seen in confluent cultures. I examined the possibility that PADPRS may play a role in the modulation of prolyl hydroxylase throughout the growth of cells in culture. In these experiments, prolyl hydroxylase activity was determined in cultures of 22 w fetal human dermal fibroblasts as a function of cell proliferation. In order to examine if PADPRS is involved, cultures were treated with ascorbate, or with ascorbate and inhibitors of PADPRS.

As seen in Fig. 6.1, the highest specific activity of prolyl hydroxylase was seen 3 d after the cells were plated. These data are based on the pooling of several dishes in order to obtain sufficient numbers of cells to measure the enzyme. The activity of enzyme per mg cellular protein decreased in a remarkable way on day 4 in culture and after this point, the activity essentially trailed the growth curve. The activity reached another maximum, after the cells became confluent, however, the enzyme once again reached very low levels in the third day of confluence on the eighth day in culture.

When the cells were treated with 0.35 mM ascorbate, the enzyme levels were observed to be nearly doubled compared to the controls, at each time point examined (Figs. 6.2, 6.7). These data suggest that ascorbate can modulate the activity of prolyl hydroxylase in growing cell cultures. In order to examine if this modulation also involved PADPRS, cultures were maintained in the presence of ascorbate and 3AB, 6AN or ascorbate with 3AB and 6AN. As seen in Figs. 6.3, 6.4, 6.5 and 6.6, the inhibitors of PADPRS abolished the effect of ascorbate at each time point.

These observations confirm my concept that the expression of prolyl hydroxylase is mediated by PADPRS.

Fig. 6.1
Culture Protein Content, mg x 10²
Prolyl Hydroxylase Activity
DPM ³H/mg Protein
Days in culture

Fig. 6.2
Culture Protein Content, mg x 10²
Prolyl Hydroxylase Activity
DPM ³H/mg Protein
Days in culture

Fig. 6.3
Culture Protein Content, mg x 10²
Prolyl Hydroxylase Activity
DPM ³H/mg Protein
Days in culture

Fig. 6.4
Culture Protein Content, mg x 10²
Prolyl Hydroxylase Activity
DPM ³H/mg Protein
Days in culture

Fig.6.5
Culture Protein Content, mg x 10²
Prolyl Hydroxylase Activity
DPM ³H/mg Protein
Days in culture

Fig. 6.6
Culture Protein Content, mg x 10²
Prolyl Hydroxylase Activity
DPM ³H/mg Protein
Days in culture

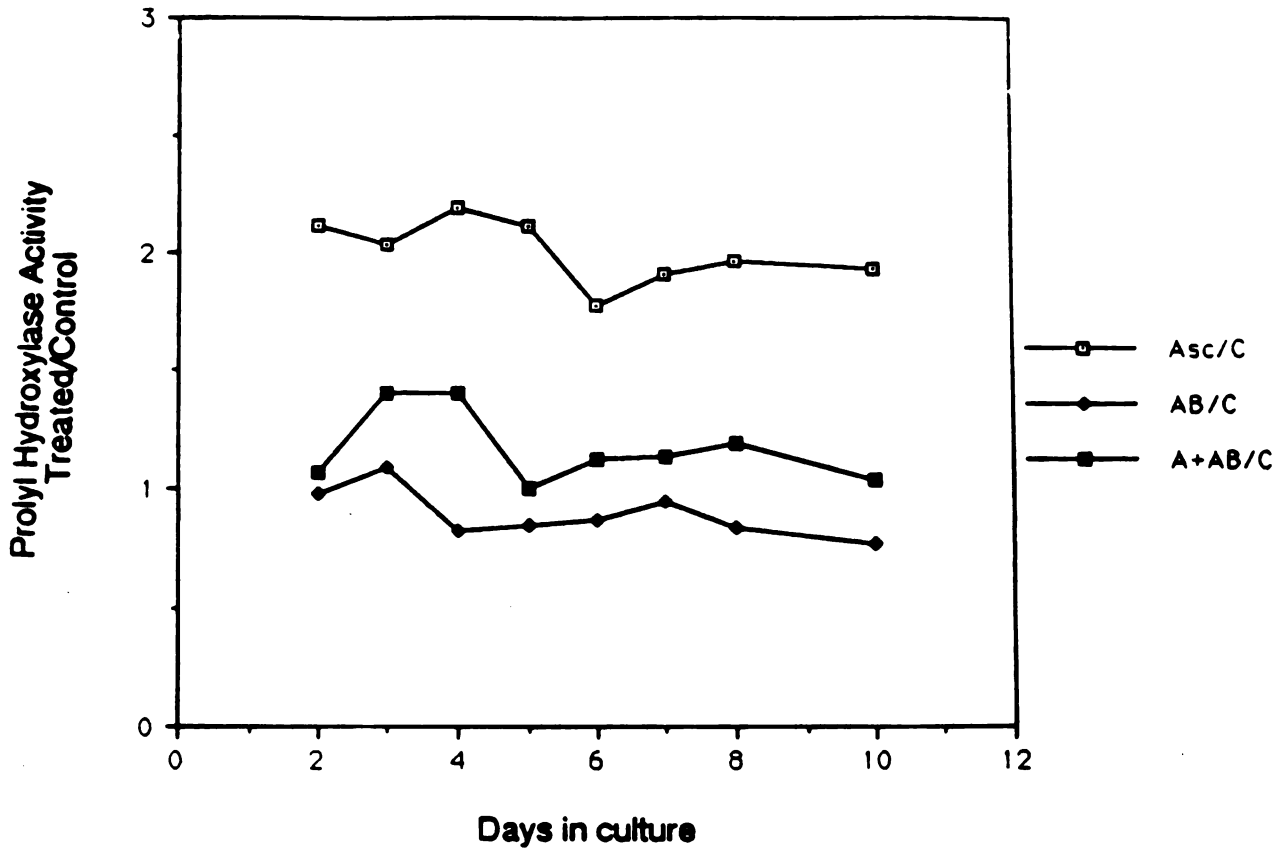


Fig. 6.7 Effect of ascorbate on prolyl hydroxylase as a function of cell proliferation. 0.35 mM ascorbate increased the enzyme activity nearly two fold at each time point (Asc/C). 2.0 mM 3AB decreased the enzyme activity to nearly the control level (3AB+Asc/C). 2.0 mM 3AB itself down-regulated prolyl hydroxylase activity (3AB/C).

Chapter 7: Modulation of Prolyl Hydroxylase During Fetal Development.

The synthesis of collagen is modulated as a function of development and aging, with the highest rates seen in the young and decreasing to very low levels in the older animal (4-6) Human fetal fibroblasts synthesize collagen in higher amounts than do post-natal fibroblasts (7). The levels of prolyl hydroxylase, a crucial enzyme in collagen synthesis, co-modulate with collagen synthesis as a function of age (8-10). Although previous studies (31) have shown an age dependent decrease in prolyl hydroxylase, the relationship of the enzyme to early developmental stages in humans has not been examined. PADPR is an important regulatory mechanism in development. We considered the possibility that PADPR may play a role in the age and development related modulation of prolyl hydroxylase.

In order to confirm this hypothesis, we examined the effect of inhibitors of PADPRS on both ascorbate-stimulated or unstimulated production of prolyl hydroxylase in human dermal fibroblasts derived from various fetal gestational ages.

Effect of Ascorbate on Prolyl Hydroxylase in Fetal Fibroblasts: As seen in Fig. 7.1, ascorbate had quite different effects on prolyl hydroxylase levels in early (<20 w) and late (>20 w) gestation fibroblasts. Fibroblasts from 11, 13, 16, 17, 18, and 19 w gestation

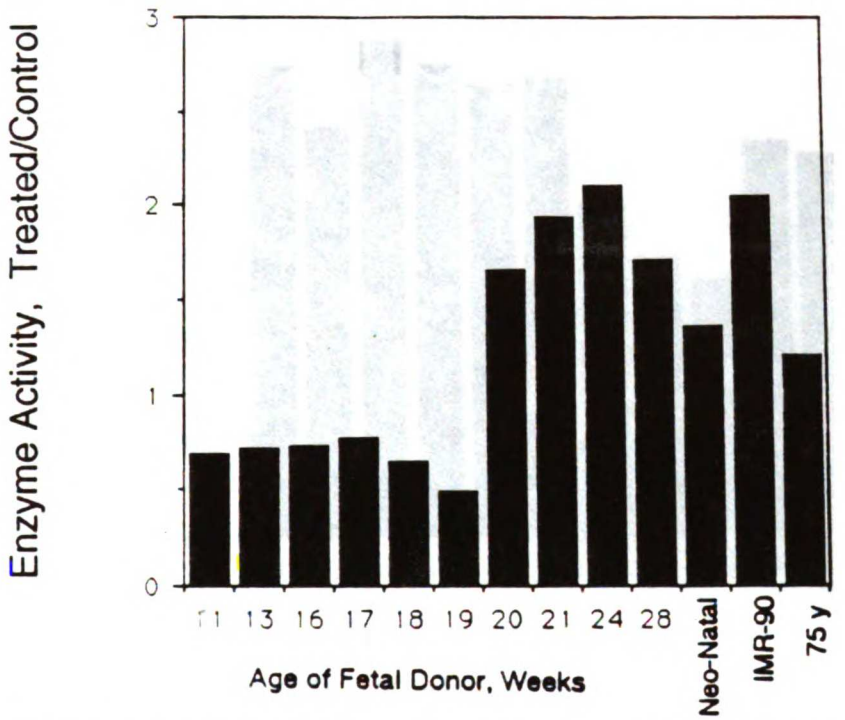
showed decreased prolyl hydroxylase activity, whereas fibroblasts from 20, 21, 24, 28, 30, 32 and 34 w gestation showed the adult-like pattern of stimulation of enzyme activity. The reason for this difference was not examined in the present studies; however, it is possible to explain this behavior by lower levels of oxidant defense, e.g. SOD, in the early developmental stages, as suggested by other workers (24, 25).

Effect of Inhibitors of PADPRS on: In general, inhibitors of PADPRS reversed the effect of ascorbate on fetal fibroblasts. As seen in Figs. 7.2 and 7.3, exposure of fetal fibroblasts to 3AB and 6AN at the same time as ascorbate, caused the levels of prolyl hydroxylase in fetal fibroblasts of gestational age <20 w to become comparable to controls. In contrast, the levels of prolyl hydroxylase in >20 w gestation fibroblasts which were elevated by ascorbate, showed a decrease to below control levels.

In contrast to neonatal, IMR-90 and adult cells, fetal cells responded to inhibitors of PADPRS by lowering the levels of prolyl hydroxylase (Table 7.1). This is consistent with the idea that PADPRS levels are much higher in developing tissues (7).

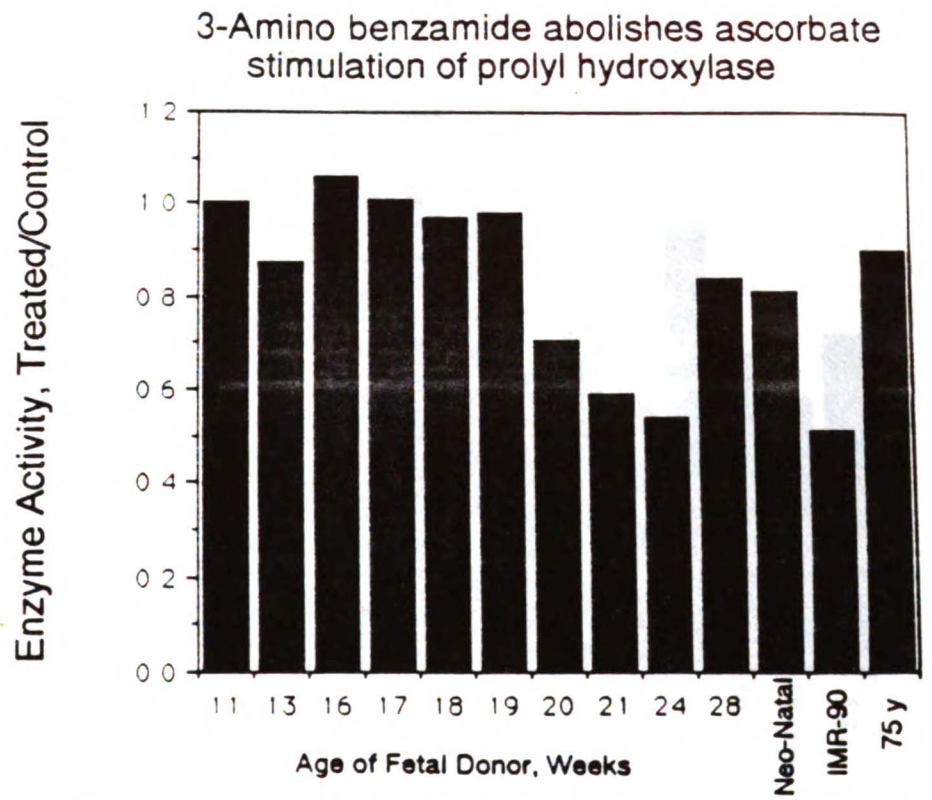
These results suggest that PADPRS is involved in the regulation of prolyl hydroxylase in the ealy developmental stages in the human. It is interesting that fibroblasts of gestational age of 20 w and above exhibit adult like behavior towards ascorbate.

Ascorbate stimulates Activity of prolyl hydroxylase



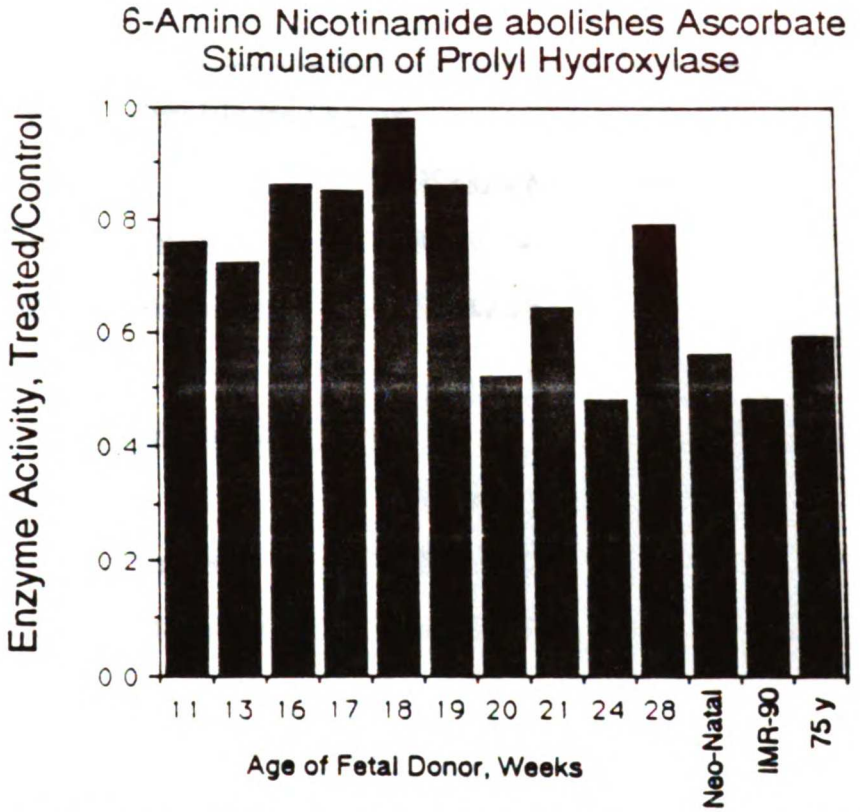
Stimulation of prolyl hydroxylase by 0.35 mM ascorbate in fetal, neo-natal, IMR-90 and 75 y fibroblasts.

Fig. 7.1



The ascorbate mediated modulation of prolyl hydroxylase activity in fetal, neo-natal, IMR-90 and 75 y fibroblasts is abolished by 3-amino benzamide suggesting that a PADPRS mediated event may be involved in the modulation of the enzyme.

Fig. 7.2



6-Amino nicotinamide abolishes the effect of ascorbate on prolyl hydroxylase activity, confirming the observations with 3-amino benzamide. These observations support our conclusion that PADPRS-mediated events may regulate prolyl hydroxylase activity in fibroblasts.

Fig. 7.3

Table 7.1: Effect of PADPRS inhibitors on prolyl hydroxylase activity

Cell type and experiment	Enzyme activity dpm ^{3}H/mg protein x 10^{-4}	Treated/Control (%)
Fetal dermal fibroblasts (11 w)		
Control	0.99±0.08	
+3-aminobenzamide	0.84±0.06	-15
+6-aminonicotinamide	0.57±0.06	-42
Fetal dermal fibroblasts (13 w)		
Control	0.92±0.16	
+3-aminobenzamide	0.85±0.05	-24
+6-aminonicotinamide	0.61±0.10	-45
Fetal dermal fibroblasts (16 w)		
Control	2.11±0.07	
+3-aminobenzamide	2.04±0.02	-3
+6-aminonicotinamide	1.29±0.11	-39
Fetal dermal fibroblasts (17 w)		
Control	2.23±0.09	
+3-aminobenzamide	2.24±0.09	+1
+6-aminonicotinamide	1.47±0.05	-34

Fetal dermal fibroblasts (18 w)		
Control	1.81±0.14	
+3-aminobenzamide	1.85±0.16	+2
+6-aminonicotinamide	1.18±0.12	-35
Fetal dermal fibroblasts (19 w)		
Control	2.26±0.22	
+3-aminobenzamide	1.89±0.11	-17
+6-aminonicotinamide	0.67±0.05	-70
Fetal dermal fibroblasts (20 w)		
Control	1.13±0.08	
+3-aminobenzamide	1.14±0.07	-15
+6-aminonicotinamide	0.87±0.04	-34
Fetal dermal fibroblasts (21 w)		
Control	2.38±0.08	
+3-aminobenzamide	2.01±0.07	-15
+6-aminonicotinamide	1.72±0.06	-28
Fetal dermal fibroblasts (24 w)		
Control	2.12±0.06	
+3-aminobenzamide	1.99±0.07	-6
+6-aminonicotinamide	1.66±0.02	-21

Fetal dermal fibroblasts (28 w)		
Control	2.05±0.09	
+3-aminobenzamide	2.04±0.02	-1
+6-aminonicotinamide	1.53±0.14	-36
IMR-90 fibroblasts		
Control	1.68±0.09	
+3-aminobenzamide	1.52±0.09	-9
+6-aminonicotinamide	1.05±0.22	-37
Neo-natal fibroblasts (foreskin)		
Control	1.99±0.05	
+3-aminobenzamide	2.02±0.04	+2
+6-aminonicotinamide	1.05±0.01	-47
Adult dermal fibroblasts (75 y)		
Control	1.80±0.16	
+3-aminobenzamide	2.38±0.17	+32
+6-aminonicotinamide	1.22±0.29	-32

All cells were used between 5th and 8th passages. No additions were made in the control. in order to examine the effect of PADPRS inhibitors on prolyl hydroxylase activity, the cultures were exposed to 2.0 mM 3-aminobenzamide and 2.0 mM 6-aminonicotinamide. The cells were harvested 24 h after the addition of inhibitors of PADPRS. Prolyl hydroxylase activity was determined as described elsewhere.

Chapter 8: Effect of Phorbol Ester and Cholera Toxin on Fibroblast Prolyl Hydroxylase Levels.

If PADPR is involved in the modulation of prolyl hydroxylase, it should be possible to increase its activity by agents which increase PADPRS. Two such agents are PMA and cholera toxin. These agents regulate many cellular biochemical events through mechanisms involving PADPR.

Phorbol Ester: Increased polyADP-ribosylation in the nucleus has been implicated in the action of PMA (68). PMA can produce a pro-oxidant state in several cell types (37) and also cause an increase in poly ADP-ribosylation in mouse and human fibroblast cultures (74). These reaction involve active oxygen formation (39,41).

Cholera Toxin: Cholera toxin acts through a mechanism involving polyADP-ribosylation of membrane-associated proteins involved in signal transduction (66,67,84). Cholera toxin functions as an enzyme and catalyzes the transfer of ADP-ribose from intracellular NAD^+ to the α-subunit of guanine nucleotide binding protein (G-protein) and lead to persistent activation of PADPRS (85). Cholera toxin induced polyADP-ribosylation is inhibited by inhibitors e.g. as nicotinamide which also inhibit nuclear polyADP-ribosylation.

Studies with ascorbate strongly suggested the involvement of PADPR in the regulation of prolyl hydroxylase. I hypothesized that phorbol ester and cholera toxin stimulation of PADPR may also regulate

prolyl hydroxylase activity. In order to confirm this hypothesis, I examined the effect of phorbol ester and cholera toxin on the activity of prolyl hydroxylase.. I also used PADPRS inhibitors to confirm the role of PADPR in the modulation of prolyl hydroxylase by PMA and cholera toxin.

PMA Increased Prolyl Hydroxylase Activity: Exposure of confluent cultures of neonatal dermal fibroblasts to PMA in a concentration range of 1-50 ng/ml resulted in a linear relationship between PMA and the enzyme (Fig. 8.1). At 50 ng/ml PMA, the enzyme activity was nearly 65% greater in the treated cells than in controls. At 100 ng/ml PMA, the stimulation was decreased, wih the treated cells showing approximately 40% higher activity. Even at 200 ng/ml, the activity was elevated, although only about 15%. The increase in prolyl hydroxylase was abolished by inhibitors of PADPRS (Table 8.1) confirming that the effect of PMA was related to PADPR activity.

Cholera Toxin Elevated Prolyl Hydroxylase Activity: When confluent cultures of neonatal dermal fibroblasts were exposed to cholera toxin, they displayed a concentration dependent increase in prolyl hydroxylase activity (Fig. 8.2). A significant increase, 25%, was seen at 10 ng/ml cholera toxin. The largest incease was seen in the presence of 50 ng/ml cholera toxin. At 100 ng/ml, the activity was almost the same as controls, and at 300 ng/ml, the activity was only about 70% of the control. This decrease may be due to the reported (61) cytotoxicity of high levels of this substance. The reason for the toxicity was not examined in my experiments. The increase in prolyl

hydroxylase was abolished by inhibitors of PADPRS (Table 8.2). These observations suggest that PADPR may be involved in the modulation of prolyl hydroxylase by cholera toxin.

There are no other reports on the effect of PMA and cholera toxin on the levels of prolyl hydroxylase. These studies support the hypothesis that PADPR plays a role in the modulation of prolyl hydroxylase.

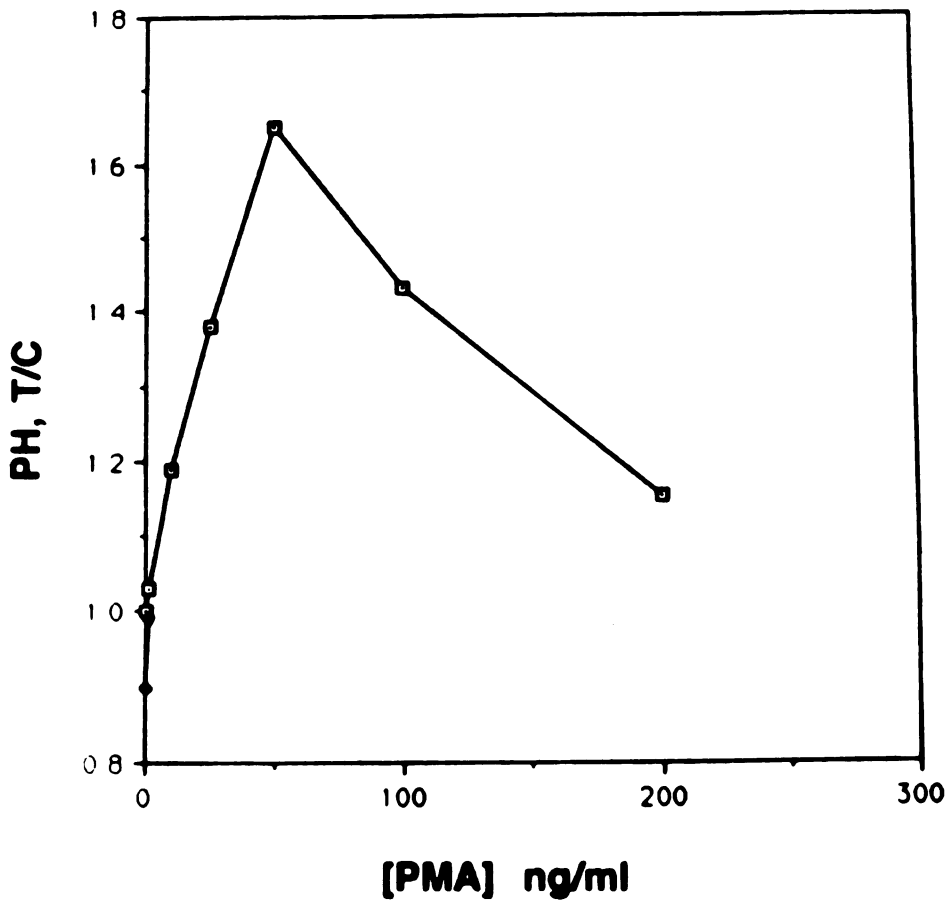


Fig. 8.1 Phorbol ester (PMA) markedly increased the activity of prolyl hydroxylase at 50 ng/ml

Table 8.1 The Effect of PADPRS Inhibitors on the Modulation of Prolyl hydroxylase by Phorbol Ester.

Treatment	Prolyl Hydroxylase Activity 10^{-4} x d.p.m. ^{3}HHO/mg protein	% Change over control	% Change over treated[a]
Control	2.62±0.26	-	-
Ascorbate, 0.3 mM	3.41±0.45	+30	
Ascorbate, 0.3 mM + 3-Aminobenzamide, 2.0 mM	2.95±0.07	+13	-13
Ascorbate, 0.3 mM + 6-Aminonicotinamide, 2.0 mM	2.68±0.12	+2	-21
Phorbol ester, 66.5 nM	3.79±0.45	+ 45	
Phorbol ester, 66.5 nM + 3-Aminobenzamide, 2 mM	2.72±0.23	+ 4	-28
Phorbol ester, 66.5 nM + 6-Aminonicotinamide, 2 mM	2.18±0.24	-17	-42
Phorbol ester, 66.5 nM + Benzamide, 2 mM	2.66±0.23	-2	-30

Phorbol ester, 66.5 nM	3.04±0.27	+16	-20
+Nicotinamide, 1.0 mM			
Phorbol ester, 66.5 nM	2.61±0.19	0	-33
+ Theophylline, 1.0 mM			
Phorbol ester, 66.5 nM	2.46±0.09	-6	-35
+ 2-Acetyl pyridine, 1 mM			
Phorbol ester, 66.5 nM	2.93 ±0.18	+12	-23
+ Thymidine, 1 mM			

Confluent cultures were treated for 24 h with the indicated additives and prolyl hydroxylase activity determined as described in the Methods.

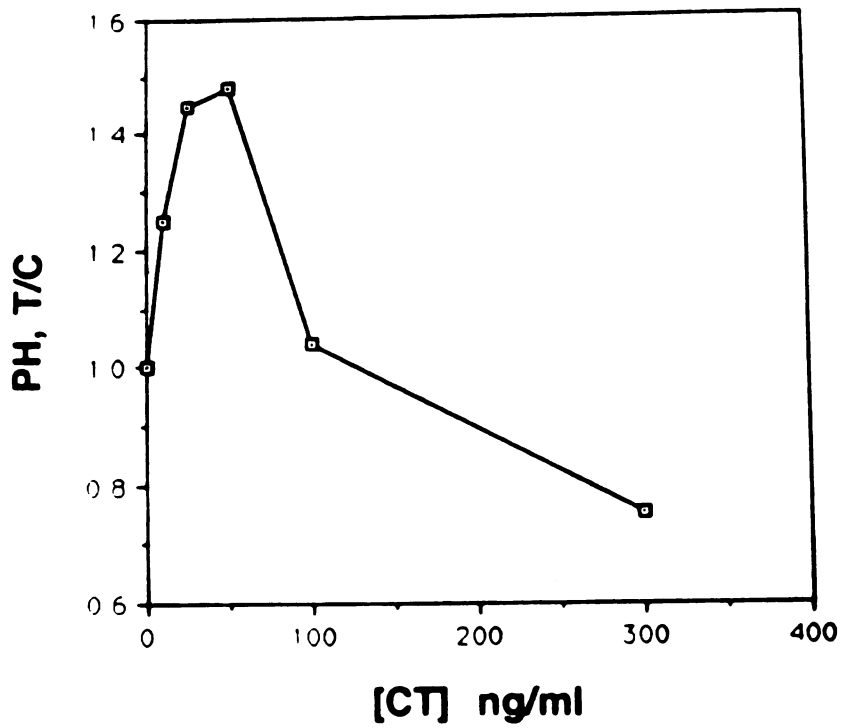


Fig. 8.2 Cholera toxin (CT) dramatically increased the activity of prolyl hydroxylase at 50 ng/ml

Table 8.2: Cholera Toxin Increases Prolyl Hydroxylase.

Treatment	Prolyl Hydroxylase Activity 10^{-4} x d.p.m. ^{3}HHO/mg protein	% Change over control	% Change over treated
Control	2.62±0.26	-	-
Cholera toxin, 50 ng/ml	3.48±0.17	+33	
Cholera toxin, 50 ng/ml + 3-Aminobenzamide, 2.0 mM	2.74±0.18	+5	-21
Cholera toxin, 50 ng/ml 6-Aminonicotinamide, 2.0 mM	2.72±0.23	+4	-22
Cholera toxin, 50 ng/ml + Benzamide, 1.0 mM	2.89±0.18	+10	-32
Cholera toxin, 50 ng/ml + Nicotinamide, 1.0 mM	2.79±0.34	+6	-20
Cholera toxin, 50 ng/ml + Theophylline, 1.0 mM	2.37±0.12	-10	-32
Cholera toxin, 50 ng/ml + 2-Acetyl pyridine, 1.0 mM	2.43±0.15	-7	-30

Cholera toxin, 50 ng/ml	2.45±0.34	-6	-30
+ Thymidine, 1.0 mM			

Experimental conditions were the same as described in the legend to Table 1.

Chapter 9. The Inhibitor of Protein Kinase C Affected on Prolyl Hydroxylase Activity

PMA and cholera toxin are not only stimulators of PADPR (71,72,74), but they are also activators of protein kinase C (PKC) (73). Many experimental results point to the central role of PKC as a regulator of cellular processes. Its activation is a crucial event in transmembrane signalling of numerous extracellular stimuli (86). PKC is also considered to be the receptor for PMA which can bind to the diacylglycerol binding site and directly activate PKC without the requirement of phosphorylation (87,88).

Effect of Inhibiting PKC on Prolyl Hydroxylase Activity: In order to examine if PKC participates in the regulation of prolyl hydroxylase, an inhibitor of PKC, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) (Iso) (89) was used in conjunction with ascorbate, PMA and cholera toxin in confluent cultures of neonatal dermal fibroblasts. As seen in Table 9.1, the inhibitor blocked the increase in prolyl hydroxylase induced by all three stimulants.

These results suggest that PKC may be involved in the modulation of prolyl hydroxylase.

Table 9.1: Protein Kinase C is Involved in the Modulation of Prolyl Hydroxylase.

Treatment	Prolyl Hydroxylase Activity 10^{-4} x d.p.m. ^{3}HHO/mg protein	% Change over control	% Change over treated
Control	2.10±0.17	-	-
Ascorbate, 0.3 mM	3.68±0.22	+ 75	
Phorbol ester, 66.5 nM	3.35±0.34	+ 60	
Cholera toxin, 50 ng/ml	2.82±0.49	+ 34	
PKC Inhibitor*, 50 μM	1.55±0.06	- 27	
Ascorbate, 0.3 mM + PKC Inhibitor, 50 μM	1.90±0.19	-10	-48
Phorbol ester, 66.5 nM + PKC Inhibitor, 50 μM	1.81±0.24	- 15	-47
Cholera toxin, 50 ng/ml + PKC Inhibitor, 50 μM	1.86±0.23	- 12	-44

* 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine. Experimental conditions are described in Table 1

Chapter 10: The Inhibition of ADP-ribosylation of G-protein Affect Prolyl Hydroxylase Activity.

PMA and cholera toxin markedly increase the activity of prolyl hydroxylase. The effects of PMA and cholera toxin are also involved in the G-protein transduction signalling pathway (90). The stimulation of G-protein mediated signalling pathways in the presence of PMA and cholera toxin is confirmed by the abolition of the effects of these agents by pertussis toxin. Pertussis toxin interferes with G-protein function by catalyzing the ADP-ribosylation of the α-subunit of Gi-protein (Giα) (60). This decreases the ability of this G-protein to interact with its receptor. In contrast, the ADP-ribosylation of Gsα by cholera toxin results in the stimulation of G-protein transduction activities mediated by receptors (58). The inhibition of biological events induced by cholera toxin suggests the involvement of the G-protein transduction pathway. Because we observed the modulation of prolyl hydroxylase by cholera toxin and PMA, both of which cause cellular changes involving the signalling pathway, we examined the participation of G-protein in the modulation of prolyl hydroxylase.

Effect of Pertussis Toxin on the Increase in Prolyl Hydroxylase in the Presence of Ascorbate, PMA and Cholera Toxin: Fibroblasts were exposed to 50 ng/ml PMA, or 50 ng/ml cholera toxin. As seen in Table10.1, both of these agents markedly increased the activity of prolyl hydroxylase. Pertussis toxin (25 ng/ml) abolished the increase induced by these agents. In order to determine if similar

mechanisms are involved in the stimulation of prolyl hydroxylase by ascorbate, the effect of pertussis toxin was examined in fibroblasts treated with 0.35 mM ascorbate. As seen in Table 10.1, the effect of ascorbate was also abolished by pertussis toxin.

Table 10.1: Involvement of G-Protein in the Modulation of Prolyl Hydroxylase.

Treatment	Prolyl Hydroxylase Activity 10^{-4} x d.p.m. ^{3}HHO/mg protein	% Change over control	% Change over treated
Control	2.62±0.26	-	-
Pertussis toxin, 25 ng/ml	2.12±0.22	-19	
Ascorbate, 0.3 mM	3.41±0.45	+30	
Ascorbate + Pertussis toxin, 25 ng/ml	2.67±0.13	+2	-26
Phorbol ester, 66.5 nM	3.79±0.45	+45	-
Phorbol ester + Pertussis toxin , 25 ng/ml	2.22±0.28	-15	-42
Cholera toxin, 25 ng/ml	3.80±0.34	+45	-
Cholera toxin + Pertussis toxin, 25 ng/ml	2.28±0.30	-13	-40

Experimental conditions are described in the legend to Table 1.

Chapter 11: Discussion

Collagen synthesis plays an important in the wound healing process. It modulates biological behavior of cells and determines the major biomechanical properties of all tissues. The hydroxylation of proline by prolyl hydroxylase is a crucial step in collagen biosynthesis (91). The activity of prolyl hydroxylase usually reflects the potential of collagen synthesis and has been shown to increase in conditions where elevated amounts of collagen are produced (92), for example, in inflammation, aging, cell proliferation, ascorbate, tissue oxygenation, certain superoxide-generating drugs including Bleomycin (6,28) and paraquat (18). The mechanism by which these stimulations take place is not clearly understood.

Since the original observations of PADPRS by Chambon et al. (93,94), several roles have been proposed for this enzyme (95-98). It was shown that poly ADP-ribose synthesis correlates with the differentiation of chick mesodermal cells into chondrogenic cells. PADPRS was observed to be increased in differentiating amphibian oocytes (93), and in mouse ova (94). This enzyme activity was also detected in the rat brain at all stages of development (95). Previous studies in this laboratory have shown that increased activity of prolyl hydroxylase and production of collagen occurs in response to environmental toxins, such as ozone (30), benzo(a)pyrene (99), or superoxide-generating drugs such as

Bleomycin (28). The increase in prolyl hydroxylase was accompanied by an increase in PADPRS in these experiments.

PADPR is a necessary component in the control of gene expression, cell differentiation, tissue development and repair (98), and this reaction is also a link between genetic (gene expression) and epigenetic (temporal programmed gene expression) mechanisms (19,100,101).

Our present observations, that fibroblasts prolyl hydroxylase activity is regulated by PADPRS during different stages of proliferation, as well as in the stationary phase, are consistant with gene expression (19). While genes for prolyl hydroxylase are present in fibroblasts at all stages of proliferation, they are not maximally expressed at each stage except in the presence of superoxide generators (such as ascorbate, phorbol ester etc.) which act as the switching mechanism. The constitutive levels of prolyl hydroxylase were increased dramatically by ascorbate between the initiation of the proliferative stage and confluence, and this increase was abolished by PADPRS inhibitors.

While genetic mechanisms deal with the transfer of genes between parent cells and their progeny, epigenetic mechanisms regulate the temporal and spatial aspects of gene expression as a part of tissue differentiation and morphological development. According to the above mechanisms, this increased prolyl hydroxylase activity is an example of epigenetic control. Therefore, we examined the effects

of PADPRS on the prolyl hydroxylase activity in human fetal, neonatal and adult skin fibroblast cultures. If PADPRS is a part of the regulatory pathway for collagen and prolyl hydroxylase, its activity should be modulated by agents that can increase collagen synthesis. Ascorbate is an oxygen free radical generator and it increased collagen synthesis and prolyl hydroxylase activity in fibroblast cultures (32,33). We examined the effect of ascorbate on PADPRS activity in cultures of neonatal human (foreskin) fibroblast. As seen in Fig.5.1, PADPRS activity was increased by 50% within 30 minutes after exposure to 0.35 mM ascorbate. The expected increase in prolyl hydroxylase activity was observed on addition of ascorbate to neonatal human dermal fibroblast, IMR-90, and adult fibroblast culture, as seen in Table 5.1. This stimulatory effect of ascorbate on prolyl hydroxylase activity was abolished by inhibitors of PADPRS in all cells. These results demonstrate that PADPRS is obviously involved in the regulation of prolyl hydroxylase activity, and furthermore, that it also controls collagen synthesis. These findings may explain how PADPR caused by different conditions which have a common feature, the so-called "pro-oxidant" state. These results may also provide a possible mechanism for PADPRS modulation of prolyl hydroxylase activity and collagen synthesis at the epigenetic level. The marked decrease of prolyl hydroxylase activity by PADPRS inhibitors suggests a key site for the regulation of collagen synthesis, and it may explain the differences of prolyl hydroxylase activity in fibroblast derived from fetal and adult tissues.

Ageing is considered another biological factor that regulates cell differentiation, macromolecular turnover and tissue repair (7). Collagen synthesis is down-regulated as part of the aging process. According to Uitto (8), the highest rates of collagen synthesis are observed in fetal skin and decrease with age. The activities of prolyl hydroxylase involved in the post-translational modulation of collagen were also similarly decreased in ageing human skin (102). Hence, studies on PADPR may help us in understanding the mechanisms involved in the molecular alterations, specifically in PADPR modification as a control of chromatin conformation and gene transcription occurring as part of the aging peocess. Thakur and Prasad (45) reported their findings on the ADP-ribosylation of HMG-protein and its modulation by different effectors in the liver of young and old rats. They find a decrease in ADP-ribosylation of HMG proteins in old age. Similar results were reported by Bizec (103) that poly ADP-ribose levels decrease with aging. Our results suggest a possible mechanism to explain the down-regulation of collagen synthesis in aging.

My studies also provide a mechanism for the difference in fetal and adult wound healing. It has been suggested that fetal wound healing may not involve collagen synthesis and may instead depend on the deposition of a proteoglycan-rich matrix as a template for subsequent tissue reconstruction (104). However, my present observations suggest that fetal cells are rich in prolyl hydroxylase, an important component of collagen synthesis. This is consistent with

the highly dynamic state of fetal tissue and the higher rates of macromolecular turnover. The decrease in enzyme activity after 20 w gestation suggested that mechanisms related to aging in the context of collagen synthesis and wound healing may be initiated during gestation. These is a specific point at the 20 w gestational landmark, which represents a remarkable changes in cell biological behavior, related to collagen synthesis. A transition from the fetal to the adult type of wound healing in human may occur at that time.

In order to stimulate PADPRS in modification of protein, such as, collagen synthesis, the enzyme activity must be increased by some inducing agents. Ascorbate is an oxygen free radical generator and our studies show that it can induce increased PADPR. Our previous studies showed that prolyl hydroxylase activity and collagen synthesis were stimulated by active oxygen (O_2^-) in lung fibroblasts (30) and in hepatocytes (17). Our present experiments with fibroblasts from fetal skin of various gestational ages, showed that the cells did not require ascorbate to activate prolyl hydroxylase before 20 w gestation. This is in marked contrast to the observation that post-natal and adult tissues and cells appeared to have a strict requirement for ascorbate for the stimulation of prolyl hydroxylase activity (105).

The stimulation of PADPRS activity by PMA (39), and cholera toxin (58) has been well documented. In order to study the role of PADPR in the regulation of prolyl hydroxylase, we examined enzyme activity in the presence of these agents. The results of this experiment

showed that PMA and cholera toxin increased prolyl hydroxylase in a concentration dependent manner (Figs 7.1 and 7.2), and this increase was abolished by inhibitors of PADPR (tables 7.1 and 7.2).
PMA and cholera toxin also participate in generating active oxygen as suggested by Cerutti (71,72) and Konno et al. (68). Cerutti's observations showed that PMA stimulated the level of PADPRS by active oxygen in mouse and human fibroblasts. Similar results were reported by Singh, that PMA induced the PADPR mediated at least in part by active oxygen e.g. superoxide in human epidermal carcinoma A431 cells (40). Cholera toxin, c-AMP and superoxide enhanced the expression of cell-surface antigen on W31 rat fetus transformed-fibroblasts by the same mechanisms (106). These observation may explain the effect of PMA and cholera toxin on PADPR through the so-called "pro-oxidant" state mechanism.

The effects of PMA and cholera toxin on PADPR have been well established (41). PADPRS in this reaction is a link between external signals and gene expression (34,35). This enzyme is also a specific substrate for PKC in the phosphorylation of proteins (107). Both PMA and cholera toxin are known as PKC stimulators. PKC acts through the activation of cyclic nucleotide-dependent signalling pathways (108). These mechanisms are involved in the modification of membrane-bound G-protein by PKC. However, pertussis toxin interferes with the mechanism of PKC modulation of G-protein by ADP-ribosylating the inhibitory G-protein(Gi), wherease, PMA and cholera toxin catalyze the stimulatory G-protein (Gs). In this case, pertussis toxin is regarded as evidence for the participation of G-

protein and PKC in the signalling pathway (60). In order to examine if G-protein and PKC-mediated mechanism may play a role in the modulation of prolyl hydroxylase, we studied the effects of pertussis toxin in the presence of ascorbate, PMA and cholera toxin. The results showed that pertussis toxin abolished the stimulation of these agents on prolyl hydroxylase. These observations support the involvement of G-protein-mediated signalling pathways in the modulation of prolyl hydroxylase.

PKC plays an important role in the G-protein mediated signalling pathway and is also a major factor of growth control in the wound healing processes (69). PKC is directly activated by PMA and acts as a receptor for the tumor promotor (73). I used H-7, an inhibitor of PKC to block the stimulatory effects of ascorbate, PMA and cholera toxin on prolyl hydroxylase. As seen in Table 9.1, this examination supported the participation of PKC in the modulation of prolyl hydroxylase activity.

Since cholera toxin and pertussis toxin catalyze different sites of G-protein (Gs and Gi) by PADPR, the inhibitors of PADPRS may also block the regulation of prolyl hydroxylase activity by the G-protein related signalling pathway (Table10.1.). Inhibitors of PADPRS abolished the effects of both cholera toxin and pertussis toxin on prolyl hydroxylase. These observations confirmed the role of G-protein-mediated signalling pathway in the modulation of prolyl hydroxylase activity.

Summary

These studies suggest that PADPR is involved in the modulation of prolyl hydroxylase. This may explain the changes in the synthesis of collagen as part of repair and remodeling. In addition, our observations also implicate the G-protein and PKC-mediated signalling pathways in regulation of prolyl hydroxylase activity.

My studies indicate that prolyl hydroxylase is subject to epigenetic control which is consistent with the idea that wound healing involves renewed ontogeny in the affected tissues. These studies also reveal that the pro-oxidant state of cells is modulated by pathobiological conditions including acute and surgical inflammation. However, chronic inflammation may not act through these regulatory pathways.

Our observations may also explain the decrease in collagen synthesis and tissue repair in aging and in chronically inflammed tissues.

References

1. Pesciotta, D.M., and Olson, B.R. (1982) The cell biology of collagen secretion., in Immunochemistry of the Extracellular Matrix, H. Furthmyr, ed., CRC Press, Boca Raton, FL, pp. 1-18.

2. Ramachandran, G.N., Bansal, M., and Bhatnagar, R.S. (1973) A hypothesis on the role of hydroxyproline in stablizing collagen structure. Biochim. Biophys. Acta. 322: 166-173.

2. Karvonen, K., Ala-Kokko, L., Pihlajanieni, T., Hekaakoski, T., Heuke, S., Gunzler, V., Kivirikko, K.I., and Savolainen, E.R. (1990) Specific inactivation of prolyl-4-hydroxylase and inhibition of collagen synthesis by oxaproline-containing peptides in cultured human skin fibroblasts. J. Biol. Chem. 265: 8415-8419.

3. Sasaki, T., Holeyfield, K.C., and Uitto, J. (1987) Doxorubicin-induced inhibition of prolyl hydroxylation during collagen biosynthesis in human skin fibroblast cultures. J. Clin. Invest. 80:1735-1741.

4. Kivirikko, K.I., and Myllyla, R. (1985) Post-translational processing of procollagens. Ann. NY Acad. Sci. 460: 187-201.

5. Uitto, J., and Prockop, D.J. (1974) Intracellular hydroxylation of procollagen to form triple-helical procollagen and subsequent secretion of the molecule. Eur. J. Biochem. 43: 221-230.

6. Hussain, M.Z., Giri, S.N., and Bhatnagar, R.S. (1985) Poly(ADP-ribose) synthetase activity during Bleomycin induced lung fibrosis in hamsters. Exptl. Mol. Pathol. 43: 162-176.

7. Niedermuller, H., Hofecker, G., and Skalicky, M. (1985) Changes of DNA repair mechanisms during the aging of the rat. Mechanisms of Aging and Development. 29: 221-238.

8. Uitto, J. (1986) Connective tissue biochemistry of aging dermis: Age-related alterations in collagen and elastin. Dermatol. Clin. 4: 433-446.

9. Anttinen, O.A., and Kivirikko, K.I. (1977) Age-related changes in human skin collagen galactosyl transferase and collagen-glucosyl transferase activities. Clin. Chem. Acta. 76: 95-101.

10. Burrington, J.D. (1971) Wound healing in the fetal lamb. J. Pediatr. Surg. 6: 523-528.

11. Hallock, G.G., Rice, D.C., Merkel, J.R., and DiPaolo, B.R. (1988) Analysis of collagen control in the fetal wound. Ann. Plast. Surg. 21: 310-315.

12. Adzick, N.S., and Longaker, M.T. (1991) Scarless fetal healing: therapeutic implications., in Fetal Wound Healing, N.S.Adzick and M.T.Longaker, eds, Elsevier, New York, pp: 317-323.

13. Adzick, N.S., Harrison, M.R., Glick, P.L., et al. (1985) Comparison of fetal, newborn, and adult wound healing by histologic, enzyme-histochemical and hydroxyproline determination. J. Pediatr. Surg. 20: 315-319.

14. Ehrlich, H.P. (1980) The role of connective tissue matrix in wound healing., in Growth Factors and other Aspects of Wound Healing: Biological and Clinical Inplications, A.Barbul, E. Pines, M.Caldwell, and T.K.Hunt, eds, Alan R. Liss, New York, pp: 281-293.

15. Hunk, T.K, and Halliday, B. (1980) Inflammation in wound healing: Fromlaudable pus to primary repair and beyound., in Wound Healing and Wound Infection,theory and Surgical Practice, T.K.Hunt, ed, Appleton Century Crofts, New York, pp: 281-293.

16. Hussain, M.Z., Watson, J.A., and Bhatnagar, R.S. (1987) Increased prolyl hydroxylase activity and collagen synthesis in hepatocyte cultures exposed to superoxide. Hepatology 7: 502- 507.

17. Stein, H.D., and Kaiser, H.R. (1971) Collagen metabolism in grannulating wound. J. Surg. Res. 11: 277-283.

18. Bhatnagar, R.S. (1977) Role of superoxide in oxidant induced pulmonary fibrosis. in Biochemical Effects of Envionmental Pollutants., S.D.Lee, ed, Ann Arbor Science, Ann Arbor MI, pp: 47-58.

19. Cerutti, P. (1985) Pro-oxidant states and tumor production. Science. 227: 375-381.

20. Fridovich, I. (1989) The biology of oxygen radicals: general concepts oxygen radicals and tissue injury. Proceeding of an Upjohn Symposium. 1-5.

21. Cunningham, M.L., Peak, J.G., and Peak, M.J. (1987) Single-strand DNA breaks in rodent and human cells produced by superoxide anion or its reduction products. Mutation Res. 184: 217-222.

22. Birnboim, H.C. (1990) DNA strand breaks in human leukocytes induced by superoxide anion, hydrogen peroxide and tumor promoters are repaired slowly compared to breaks induced by ionizing radiation. Carcinogenesis 7: 1511-1517.

23. Margan, A.R., Cone, R.L., and Elgert, T. (1976) The mechanism of DNA strand breakage by vitamin C and superoxide and the protective role of catalase and superoxide dismutase. Nucleic Acids Res. 3: 1139-1149.

24. Rickett, G.M.W., and Kelly, F.J. (1990) Developmental expression of antioxidant enzymes in guinea pig lung and liver. Development. 108: 331-336.

25. Allen, R.G., and Balin, A.K. (1988) Developmental changes in the superoxide dismutase activity of human skin fibroblasts are maintained in vitro and are not caused by oxygen. J. Clin. Invest. 82: 731-734.

26. Gregory, E.M., and Fridovich, I. (1973) Oxygen toxicity and superoxide dismutase. J. Bacterial. 114: 1193-1197.

27. Bhatnagar, R.S., Hussain, M.Z., and Lee, S.D. (1980) The role of collagen as a primary molecular site of environmental injury. in The molecular Basis of Environmental Toxicity., R.S.Bhatnagar, ed, Ann Arbor, Science, Ann Arbor MI, pp: 531-558.

28. Larramendy, M.L., Lopez-Larraza, D., Vidal-Rioja, L., and Bianchi, N.O. (1989) Effect of the metal chelating agent o-phenathroline on the DNA and chromosome damage induced by Bleomycin in Chinese Hamster Ovary cells. Cancer Res. 49: 6583-6586.

29. Bus, J.S., Cagen, S.Z., and Gibson, J.E. (1976) A mechanism of paraquat toxicity in mice and rats. Toxiciol. Appli. Pharmacol. 35: 501-513.

30. Hussain, M.Z., Mustafa, M.G., Ghani, Q.P., and Bhatnagar,R.S. (1985) Stimulation of poly (ADP-ribose) synthetase activity in the lungs of mice exposed to low level of Ozone. Arch. Biochem. Biophys. 241: 477-485.

31. Shinar, E., Navok, T., and Chevion, M. (1983) The analogous mechanism of enzymatic inactivation induced by ascorbate and superoxide in the presence of copper. J. Biol. Chem. 258: 14778-14783.

32. Pinnell, S.R. (1985) Regulation of collagen synthesis by ascorbic acid: A review. Yale J. Biol. Med. 58: 553-559.

33. Sandell, L.J., and Daniel, J.C. (1988) Effects of ascorbic acid on collagen mRNA levels in short term chondrocyte cultures. Connective Tissue Res. 17: 11-22.

34. Ferro, A.M., Higgins, N.P., and Olivera, B.M. (1983) Poly (ADP-ribosylation) of DNA topoisomerase. J. Biol. Chem. 258: 6000-6003.

35. Tanuma, S., and Johnson, G.S. (1983) ADP-ribosylation of nonhistone high mobility group protein in intact cells. J. Biol. Chem. 258: 4067-4070.

36. Kirsten, E., Jackowski, G., Mclick, J., Hakam, A., Decker, K., and Kun, E. (1985) Cellular regulation of poly (ADP) ribosylation of proteins. Exptl. Cell Res. 161: 41-52.

37. Singh, S., Poirier, G., and Cerutti, P. (1985) Tumor promoter phorbol-12-myristate-13-acetate induces poly (ADP)-ribosylation in fibroblasts. EMBO J. 4: 1491-1494.

38. Krupitza, G., and Cerutti, P. (1989) ADP-ribosylation of ADPR-transferase and topoisomerase I in intact mouse epidermal cell JB6. Biochemistry 28: 2034-2040.

39. Cerutti, P., Krupitza, G., and Muhlematter, D. (1987) Poly (ADP-ribosylation) of nuclear proteins by oxidant tumor promoters. Preliminary account of this work presented at the 8th International Symposium on Niacin, Nutrition, ADP-ribosylation and Cancer. Fort Worth, TX, June.

40. Singh, N. (1990) A comparative study on the effect of tumor promoter on poly ADP-ribosylation in A431 cells. Int. J. Cancer 46: 648-652.

41. Cerutti, P. (1989) Mechanisms of action of oxidant carcinogens. Cancer Detection and Prevention 14: 281-284.

42. Quesada, P., Faraone-Mennella, M.R., Jones, R., Malanga, M., and Farina, B. (1989) ADP-ribosylation of nuclear protein in rat ventral prostate during ageing. Biochem. Biophys. Res. Commun. 170: 900-907.

43. Cherney, B.W., Midura, R.J., and Caplan, A.L. (1985) Poly (ADP-ribose) synthetase and chick limb mesenchymal cell differentiation. Development Biol. 112: 115-125.

44. Tanuma, S., Johnson, L.D., and Johnson, G.S. (1983) ADP-ribosylation of chromosomal proteins and mouse mammary tumor virus gene expression. J. Biol. Chem. 258: 15371-15376.

45. Thakur, M.K., and Prasad, S. (1990) ADP-ribosylation of HMG proteins and its modulation by different effectors in the lever of ageing rats. Mechanisms of Ageing and Development. 53: 91-100.

46. Shambaugh III, G.E., Koehler, R.R., and Radosevich, J.A. (1989) Developmental pattern of poly (ADP-ribose) synthetase and

NAD glycohydrolase in the brain of the fetal and neonatal rat. Neurochem. Res. 13: 973-981.

47. Ueda, K.(1990) Poly (ADP-ribose) synthetase., in ADP-Ribosylating Toxins and G protein, J.Moss and M.Vaugham, eds, American Society for Microbiology, Washington,D.C., pp: 525-542.

48. Ueda, K., Ohashi, Y., Hatakeyama, K., and Hayaishi, O. (1983) Inhibition of DNA ligase activity by histones and its reversal by poly (ADP-ribose)., in ADP-ribosylation, DNA Repair and Cancer, M.Miwa et al.eds, Japan Sci. Soc. Press, TOKYO/VNU Science Press, Utrecht. pp: 175-182.

49. Cleaver, J.E., Bodell, W.J., Borek, C., Morgan, W.F, and Schwartz, J.L. (1983) Poly (ADP-ribose): Spectator or participant in excision repair of DNA damage., in ADP-ribosylation, DNA Repair and Cancer. M.Miwa et al.,eds, Japan Sci. Soc. Press, TOKYO/VNU Science Press, Utrecht, pp: 195-207.

50. Borek, C., Morgan, W., Ong, A., and Cleaver, J.E. (1984) Inhibition of malignant transformation in vitro by inhibitors of poly (ADP-ribose) synthesis. Proc. Natl. Acad. Sci. USA. 81: 243- 247.

51. Brac, T., Ebisuzaki, K. (1982) Poly ADP-ribosylation and friend erythroleukemic-cell differentiation: action of poly (ADP-ribose) polymerase inhibitors. Differentiation 34: 139-143.

52. Althaus, F.R., Lawrence, S.D., Sattler, G.L., and Pitot, H.C. (1982) ADP-ribosyltransferase activity in cultured hypatocytes. J. Biol. Chem. 257: 5528-5535.

53. Schraufstatter, I.U., Hyslop, P.A., Hinshaw, D.B., Spragg, R.G., Sklar, L.A., and Cochrane, C.G. (1986) Hydrogen peroxide-induced injury of cells and its prevention by inhibitors of poly (ADP-ribose) polymerase. Proc. Natl. Acad. Sci. USA. 83: 4908-4912.

54. Smets, L.A., Loesberg, C., Jamsson, M., and Rooij, H.V. (1990) Intracellular inhibition of mono (ADP-ribosylation) by meta-iodobenzyl-guanidine: specificity, intracellular concentration and effects on glucocorticoid-mediated cell lysis. Biochim. Biophys. Acta. 1054: 49-55.

55. Freissmuth, M., Casey, P.J., and Gilman, A.G. (1989) G proteins control diverse pathways of transmembrane signalling. FASEB 3: 2125-2131.

56. Gilman, A.G. (1987) G proteins: transducers of receptor-generated signals. Ann. Rev. Biochem. 56: 615-649.

57. Stryer, L., and Bourne, H.R. (1986) G proteins: A family of signal transducers. Ann. Rev. Cell Biol. 2: 391-419.

58. Obara, S., Yamada, K., Yoshimura, Y., and Shimoyama, M. (1991) Evidence for the endogenous GTP-dependent ADP-ribosylation of the a-subunit of the stimulatory guanyl-uncleotide-binding protein concomitant with an increase in basal adenylyl cyclase activity in chicken spleen cell membrane. Eur. J. Biochem. 200: 75-80.

59. Gierschik, P., Sidiropoulos, D., Spiegel, A., and Jakobs, K.H. (1987) Purification and immunochemical characterization of the major pertussis-toxin-sensitive guanine-nucleotide-binding protein of bovine-neutrophil membranes. Eur. J. Biochem. 165: 185-194.

60. Gierschik, P., Falloon, J., Milligan, G., Pines, M., Gallin, J.I., and Spiegel, A. (1986) Immunochemical evidence for a novel pertussis toxin substrate in human neutrophils. J. Biol. Chem. 261: 8058-8062.

61. Hopkins, R.S., Stamnes, M.A., Simon, M.I., and Hurley, J.B. (1988) Cholera toxin and pertussis toxin substrates and endogenous ADP-ribosyltransferase activity in Drosophila melanogaster. Biochem. Biophys. Acta. 970: 355-362.

62. Shamay, A., Pines, M., Waksman, M., and Gerlter, A. (1990) Proliferation of bovine undifferentiated mammary epithelial cells in vitro is modulated by G-protein. Mol. cellular Endocrinology 69: 217-226.

63. Collins, S., Caron, M.G., and Lefkowitz, R.J. (1992) From ligand binding to gene expression: new insights into the regulation of G-protein-coupled receptors. TIBS 17: 37-39.

64. Kahn, R.A., and Gilman, A.G. (1986) The protein cofactor necessary for ADP-ribosylation of Gs by cholera toxin is itself a GTP binding protein. J. Biol. Chem. 261: 7906-7911.

65. Fujinaga, Y., Morozumi, N., Sato, K., Tokumitsu, Y., Fujinaga, K., and Kondo,Y. (1989) A pertussis toxin-sensitive GTP-binding protein plays a role in the Go-Gi transition of rat hepatocytes following establishment in primary culture. FEBS Lett. 245: 117-121.

66. Verghese, M., Uhing, R.J., and Snyderman, R. (1986) A pertussis/cholera toxin-sensitive N protein may mediate chemoattractant receptor signal transduction. Biochem. Biophys. Res. Comm. 138: 887-894.

67. Yamamoto, T., Nakazawa, T., Miyata,T., Kaji, A., and Yokota, T. (1984) Evolution and structure of two ADP-ribosylation

enterotoxins, Escherichia coli heat-labile toxin and cholera toxin. FEBS Lett. 169: 241-246.

68. Konno, Y., Ohno, S., Akita, Y., Kawasaki, H., and Suzuki, K. (1989) Enzymatic properties of a novel phorbol ester receptor/protein kinase nPKC. J. Biochem. 106: 673-678.

69. Nishizuka, Y. (1986) Studies and perspectives of protein kinase C. Science 233: 305-312.

70. Tsukuda, M., Asaoka, Y., Sekiguchi, K., et al. (1988) Properties of protein kinase C subpecies in human platelets. Biochem. Biophys. Res. Commun. 155: 1387-1395.

71. Muchlematter, D., Larsson, R., and Cerutti, P.A. (1988) Active oxygen induced DNA strand breakage and poly ADP-ribosylation in promotable and non-promotable JB6 mouse epidermal cells. Carcinogenesis 9: 239-245.

72. Cerutti, P.A., and Trump, B.F. (1991) Inflammation and oxidative stress in carcinogenesis. Cancer Cells 3: 1-7.

73. In Molecular Biology of the Cells., B. Alberts, D. Bray, J. Lewis, M. Roff, K. Roberts, J. D.Watson eds, (1989) New york, London, pp: 1092-1094.

74. Ludwig, A., Dietal, M., Schafer, G., Muller, K., and Hilz, H. (1990) Nicotinamide and nicotinamide analogues as antitumor promoters in mouse skin. Cancer Res. 50: 2470-2475.

75. Naegeli, H., Loetscher, P., and Althaus, F.R. (1989) Poly ADP-ribosylation of proteins. J. Biol. Chem. 264: 14382-14385.

76. Ohashi, Y., Ueda, K., Hayaishi, O., Ikai, K., and Niwa, O. (1984) Induction of murine teratocarcinoma cell differentiation by suppression of poly (ADP-ribose) synthesis. Proc. Natl. Acad. Sci. USA. 81: 7132-7136.

77. Bradford, M. (1976) A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye binding. Anal. Biochem. 72: 248-254.

78. Hutton, J.J., Tappel, A.L., and Udenfriend, S. (1966) A rapid assay for collagen prolyl hydroxylase. Anal. Biochem. 16: 348-394.

79. Ghani, Q.P., and Hollenberg, M. (1978) Poly (ADP-ribose) metabolism and regulation of myocardial cell growth by oxygen. Biochem. J. 170: 387-394.

80. Schraufstatter, I.U., Hinshaw, D.B., Hyslop, P.A., Spragg,R.G., and Cochrane ,C.G. (1986) Oxidant injury of cells. J. Clin. Invest. 77: 1312-1320.

81. In Cell Culture., W.B.Jakeby, I.H.Pastan eds, (1979) Academic Press INC, New York, London, Tokyo, pp: 150-152.

82. Levene, C.I., Aleo, J.J., Prynne, C.J., and Bates, C.J. (1974) The activation of protocollagen proline hydroxylase by ascorbic acid in cultured 3T6 fibroblasts. Biochem. Biophys. Acta.338:29-36.

83. Chan, D., Lamande, S.R., Cole, W.G., and Bateman, J.F. (1990) Regulation of procollagen synthesis and processing during ascorbate-induced extracellular matix accumulation in vitro. Biochem. J. 269: 175-181.

84. In ADP-ribosylation of Protein., F.R.Althaus and C.Richter eds, (1987) Springer-Verlag, Berlin Heidelberg, New York, London, Paris, Yokyo, pp: 100-113.

85. Nishizuka, Y. (1989) Studies and prospectives of the protein kinase C family for cellular regulation. Cancer 63: 1892-1903.

86. Parker, P.J., Cousseus, L., Totty, N., et al. (1986) The complete primary structure of protein kinase C-the major phorbol ester receptor. Science 233: 853-859.

87. Ashendel, C.L., Staller, J.M., and Boutwell, R.K. (1983) Protein kinase activity associated with a phorbol ester receptor purified from mouse brain. Cancer Res. 43: 4333-4337.

88. Gill, D.M., and Meren, R. (1978) ADP-ribosylation of membrane protein catalyzed by cholera toxin: basis of the activation of adenylate cyclase. Proc. Natl. Acad. Sci. USA. 75: 3050-3054.

89. Hidaka, H., Inagaki, M., Kawamoto, S.,and Sasaki, Y. (1984) Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. Biochemistry 23: 5036-5041.

90. Gill, D.M., and Colburn, J. (1987) ADP-ribosylation by cholera toxin: functional analysis of a cellular system that stimulates the enzymic activity of cholera toxin fragment A_1. Biochemistry 26: 6364-6371.

91. Uitto, J., Halme, J., Hanuuksela, M., et al. (1976) Protocollagen proline hydroxylase activity in the skin of normal human subjects and patients with scleroderma. Scand. J. Clin. Invest. 23: 241-244.

92. Anttinen, H., Orava, S., Ryhanen, L., and Kivirikko, K.I. (1973) Assay of protocollagen lysyl hydroxylase in the skin of human

subjects and changes in the activity with age. Clin. Chem. Acta. 47: 289-294.

93. Chambon, P., Weill, J.D., and Mandel, P. (1963) Nicotinamide mononucleotide activation of a new DNA-dependent polyadenylic acid synthesizing nuclear enzyme. Biochem. Biophys. Res. Commun. 11: 39-43.

94. Chambon, P., Weill, J.D., Doly, J., Stresser, M.T., and Maudel, P. (1966) On the formation of a noval adenylic compound by enzymatic extracts of liver nuclei. Biochem. Biophys. Res. Commun. 25: 638-643.

95. Ueda, K., Ogata, N., Kawaichi, M., Inada, S., and HyaishiO. (1982) ADP-ribosylation reactions. Current Topics in Cellular Regulation 21:175-187.

96. Hilz, H., and Stone, P.R. (1976) Poly (ADP-ribose) and ADP-ribosylation of proteins. Rev. Physiol. Biochem. Pharmacol. 76:1-58.

97. Pekala, P.T., and Moss, J. (1983) Poly ADP-ribosylation of protein. Current Topics in Cellular Regulation. 22: 1-49.

98. Purnell, M.R., Stone, P.R., and Whish, W.J.D. (1980) ADP-ribosylation of nuclear proteins. Biochemical Society Transactions 8: 215-227.

99. Hussain, M.Z., Lee, S.D., and Bhatnagar, R.S. (1979) Increased aryl hydrocarbon hydroxylase and prolyl hydroxylase activity in lung organ cultures exposed to benzo[a]pyrene. Toxicology 12: 267-271.

100. Holliday, R. (1989) DNA methylation and epigenetic mechanisms. Cell Biophysis. 15: 15-20.

101. Holliday, R. (1987) The inheritance of epigenetic defects. Science 238: 163-169.

102. Balin, A.K., and Allen, R.G. (1986) Mechanisms of biologic aging. Dermatologic. Clin. 4: 347-358.

103. Bizec, J.C., Klethi, J., and Mandel, P. (1989) Regulation of protein adenosine diphosphate ribosylation in bovine lene during ageing. Ophthalmic Res. 21: 175-183.

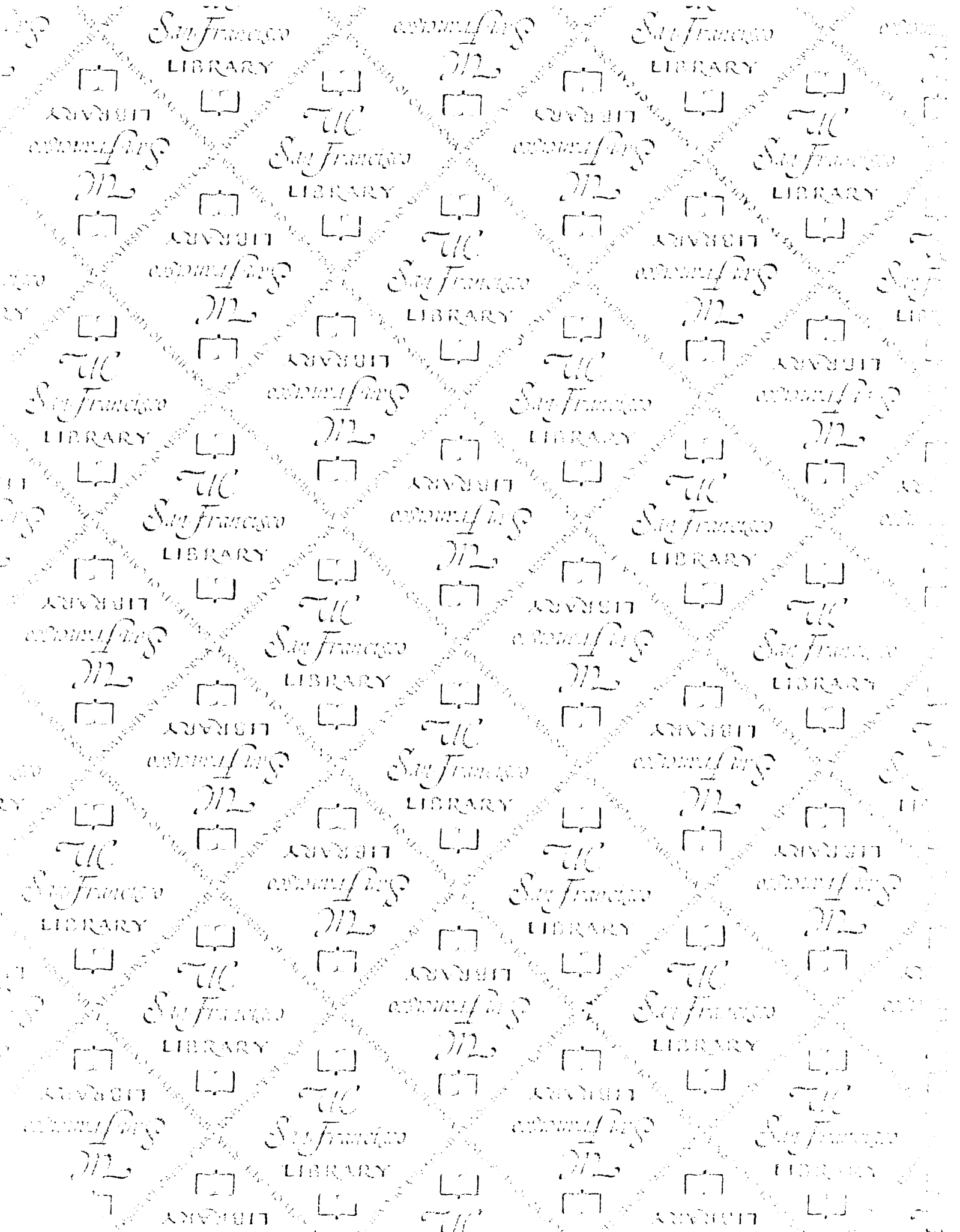
104. DePalma, R.L., Krummel, T.M., Nelson, J.M., et al. (1987) Fetal wound matrix is composed of proteoglycan rather than collagen. Surg. Forum. 38: 626-628.

105. Qian, J.J., Liu, X., Zha, J., and Bhatnagar, R.S. (1991) Inducers of poly (ADP-ribose) synthetase elevate prolyl hydroxylase activity in fibroblasts. Submitted for publication.

106. Konno, Y., Sato, N., Yagihashi, A., Kikuchi, K., et al. (1989) Heat-or stress-inducible transformation-associated cell surface antigen on the activated H-ras oncogene-transfected rat fibroblast. Cancer Res. 49: 6578-6582.

107. Nishizuka, Y. (1988) The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334: 661-665.

108. Nishizuka, Y. (1984) The role of protein kinase C in cell surface signal transduction and tumor promotion. Nature 308: 693-698.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