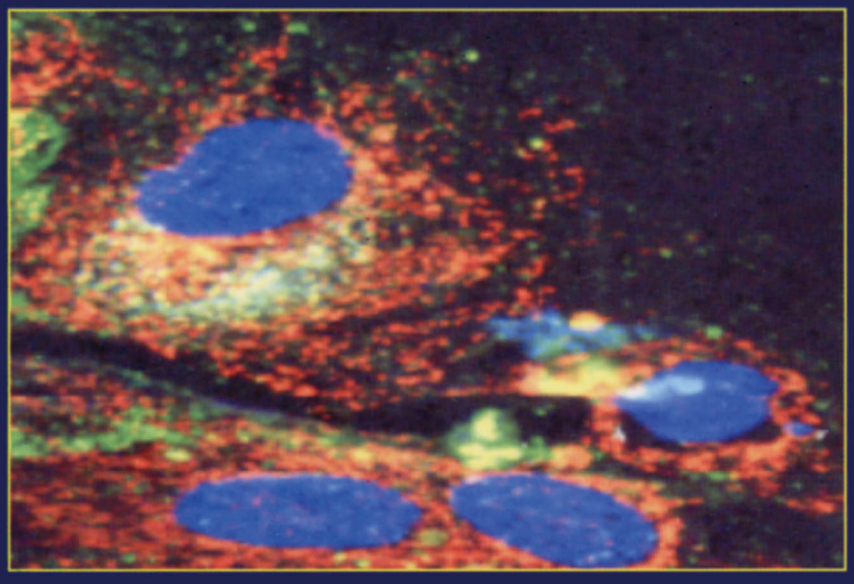
LIVER DISEASES

Biochemical Mechanisms and New Therapeutic Insights

Volume 1 and 2



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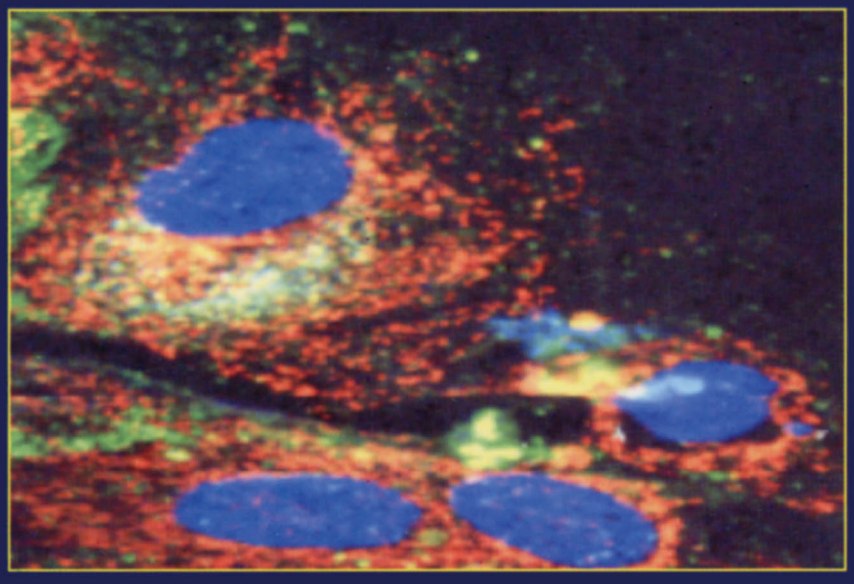
Volume 1

Pathways, Mediators and Regulation



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Volume 1

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Preface

There have been major advances in our understanding of the liver, and a growing number of mechanisms underlying liver diseases continue to pose new challenges. This book presents state-of-the-art information summarizing the current understanding of a range of liver diseases and reviews some key diagnostic and therapeutic advances.

The book constitutes a collection of selected clinical and scientific topics divided into two volumes, each divided into two sections. The first volume treats the cellular, biochemical and immunological mechanisms underlying liver diseases; the second focuses on clinical liver disease pathophysiology and related diagnostics and therapeutic insights. Collectively, the two volumes represent a broad range of important topics but are not intended as a comprehensive summary of all domains in liver research and disease.

It is hoped that the target readers—hepatologists, clinicians, researchers and academicians—will be afforded new ideas and exposed to subjects well beyond their own scientific disciplines. In addition, students and all those who wish to enlarge their knowledge of advances in the field of liver diseases should find this book a valuable source of information.

Our thanks are extended to the authors, the publisher and, most importantly, our families.

Shakir Ali
Scott L. Friedman
Derek A. Mann

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Section – I

Liver Diseases: Mediators and Regulation

1

Collagen Gene Regulation in the Hepatic Stellate Cell

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SUMMARY

Liver fibrosis has multiple causes; however, a common characteristic of fibrosis is a dramatic increase in the synthesis and deposition of extracellular matrix (ECM) proteins. This increase in ECM disrupts the architecture of the liver, resulting in altered organ morphology and function that often leads to organ dysfunction. If allowed to persist, fibrosis can progress to cirrhosis, which can lead to death. Of the numerous ECM proteins normally found in the liver, type I collagen represents the predominant ECM protein that becomes overexpressed during liver fibrosis. A preponderance of experimental evidence now indicates that the hepatic stellate cell (HSC) is the primary cell type in the liver responsible for the excess deposition of type I collagen that is seen in liver fibrosis. It is fairly well established that this increase in type I collagen by the HSC is due to both transcriptional and posttranscriptional mechanisms. This chapter has reviewed what is known about type I collagen gene regulation in the HSC.

Keywords: hepatic stellate cell; collagen gene

1. Introduction

1.1. *Liver fibrosis*

1.1.1. *Characterization of liver fibrosis*

Liver fibrosis is a wound-healing process in response to chronic liver injury. Fibrosis is characterized by a dramatic increase in the synthesis and deposition of extracellular matrix (ECM) proteins in the interstitial space. During liver fibrosis excess ECM accumulates in the space of Disse with type III collagen representing the first collagen type to increase; however, type I collagen gradually becomes the dominant collagen type deposited, which can exceed type III collagen by a 4:1 ratio and may accumulate up to 60–70% of the total collagen content in the liver (Ballardini *et al.*, 1985; Milani *et al.*, 1990; Schuppan, 1990; Ramadori *et al.*, 1998). Collagen mRNA levels can increase up to 4-fold while liver collagen protein levels can increase nearly 10–20–fold (Panduro *et al.*, 1988; Aycock and Seyer, 1989; Schuppan, 1990; Brenner *et al.*, 1993). Several other ECM constituents also increase during liver fibrosis, including laminin, fibronectin, type IV collagen, among others (Maher and McGuire, 1990; Weiner *et al.*, 1992; Jarnagin *et al.*, 1994; Milani *et al.*, 1995; Desmouliere *et al*, 1997; Richter *et al.*, 1998; Matsumoto *et al.*, 1999). The excess deposition of ECM proteins modifies the normal architecture of the liver, which can lead to organ dysfunction as a result of solute transfer being hindered to and from the hepatocytes. Over time the excess deposition of ECM eventually obliterates sinusoidal fenestrations. Along with loss of hepatocyte microvilli, nutrient and metabolite exchange

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between hepatocytes is prevented and the hepatic sinusoid leading to the physiological problems associated with active liver fibrosis. This process, called "capillarization of the sinusoids," precedes development of the symptoms associated with chronic liver disease.

If allowed to persist, liver fibrosis can eventually proceed to cirrhosis, a life threatening condition that is characterized by bridging fibrosis and if left untreated can ultimately lead to organ failure and death. Currently, the only treatment for cirrhosis is liver transplant. There are many causes of liver fibrosis that includes excessive ethanol consumption, viral hepatitis, iron or copper overload, biliary obstruction, parasitic infections including schistosomiasis, drug-induced, and autoimmune hepatitis (Fig. 1.1).

1.1.2. *Role of HSC in development of liver fibrosis*

The hepatic stellate cell (HSC) has been shown to be the main collagen-producing cell in liver fibrogenesis (Maher *et al.*, 1988; Takahara *et al.*, 1988; Milani *et al.*, 1989; Maher and McGuire, 1990; Milani *et al.*, 1990a; Milani *et al.*, 1990b; Nakatsukasa *et al.*, 1990; Schuppan, 1990; Weiner *et al.*, 1990; Tsukamoto *et al.*, 1991; Goddard *et al.*, 1998; Friedman, 2000). Located in the space of Disse in close proximity to hepatocytes on one side and endothelial and Kupffer cells on the other side, HSCs (also called fat-storing cells, Ito cells, or hepatic lipocytes) are desmine-positive, perisinusoidal cells that are the primary cell in the body responsible for vitamin A storage in the form of retinyl esters in the cell cytoplasm. These cells have the capacity to synthesize several extracellular matrix proteins and glycoproteins including at least five collagen types, fibronectin, laminin, e, tenascin, and several proteoglycans (Friedman *et al.*, 1985; Maher *et al.*, 1988; Maher *et al.*, 1989; Friedman, 1990; Loreal *et al.*, 1991; Loreal *et al.*, 1992; Méyer *et al.*, 1992; Ramadori *et al.*, 1992; Schwogler *et al.*, 1992).

Regardless of the etiology, hepatic injury induces HSCs to undergo a complex transformation or activation process into myofibroblast-like cells (reviewed in: Friedman, 2000). Following a fibrogenic stimulus, several morphological changes occur that are typically associated with HSC activation. These include loss of vitamin A stores, appearance of rough endoplasmic reticulum, and expression of smooth muscle α-actin, hence these cells are often called myofibroblast-like cells (DeLeeuw *et al.*, 1984; Mak *et al.*, 1984; Kawase *et al.*, 1986; Ogawa *et al.*, 1986; Shiratori *et al.*, 1987; Maher *et al.*, 1988; Geerts *et al.*, 1989; Ramadori *et al.*, 1990; Weiner *et al.*, 1990; Tsukamoto *et al.*, 1991; Friedman *et al.*, 1992, Knittel *et al.*, 1992). Metabolically an increase in DNA synthesis and cellular proliferation occurs with HSC activation. A dramatic increase in type I collagen expression, both at the mRNA and protein levels, occurs following HSC activation with smaller, but significant increases in types III and IV collagens (DeLeeuw *et al.*, 1984; Mak *et al.*, 1984; Kawase *et al.*, 1986; Ogawa *et al.*, 1986; Shiratori *et al*, 1987; Maher *et al.*, 1988; Geerts *et al.*, 1989; Milani *et al.*, 1989; Tsukamoto *et al.*, 1991; Friedman *et al.*, 1992; Knittel *et al.*, 1992). Expression of HSP47, a collagen-binding stress protein that acts as a collagen-specific molecular chaperone during collagen biosynthesis, is induced following HSC activation and expression is seen in the liver during fibrosis (Masuda *et al.*, 1994). Expression of all three isoforms of TGFβ, the most potent fibrogenic cytokine for HSCs, and its receptors are increased following HSC activation (Gong *et al.*, 1998; Matsuoka *et al.*, 1989; Friedman *et al.*, 1994). Furthermore, synthesis of platelet-derived growth factor-β (PDGF-β), the most potent mitogen for HSCs, is increased following HSC activation (Matsuoka *et al.*, 1989; Nakatsukasa *et al.*, 1990; Weiner *et al.*, 1992; Marra *et al.*, 1994; Masuda *et al.*, 1994). In accordance, PDGF-β receptor expression is increased in activated HSCs (Wong *et al.*, 1994; Pinzani *et al.*, 1995). With enhanced expression of PDGF and TGFβ these effectively amplify the fibrogenic response of the HSC. Expression of type IV collagenase occurs in the activated HSC. This leads to destruction of the normal hepatic basement membrane. Activated HSCs become responsive to growth factors, of which PDGF is the most potent, and to fibrogenic cytokines, of which TGFβ is the most potent. HSC activation is also associated with additional changes in the pattern of gene expression. These include synthesis of several modulators of extracellular matrix homeostasis that alters the delicate balance by increasing ECM synthesis and decreasing ECM degradation, which together advances the fibrogenic state within the liver.

The stimulus responsible for HSC activation is not well understood. It is believed that stimuli may be derived from hepatocytes, Kupffer cells, and/or sinusoidal endothelial cells. The Kupffer cell plays a critical role in the development of liver fibrosis. Depletion of Kupffer cells in the liver, using gadolinium chloride, significantly reduced fibrosis in experimental animal models of fibrosis (Adachi *et al.*, 1994). Endotoxin appears to be a key factor in activation of Kupffer cells (Thurman, 1998). Once activated, the Kupffer cell generates significant levels of highly reactive oxygen intermediates (ROIs). These molecules are believed to contribute to HSC activation either directly or through generation of lipid peroxidation products. Antioxidants block HSC activation in oxidative stress conditions where high levels of ROIs exist (Olaso and Friedman, 1998). TGFβ,

Stimuli for Fibrogenesis

Ethanol
Hepatitis B and C
Copper overload
Iron overload
Biliary obstruction
Schistosomiasis
Drug-induced
Autoimmune hepatitis

Fig. 1.1: Stimuli for liver fibrosis.

produced by activated Kupffer cells and activated HSCs, amplifies the fibrogenic response of the HSC that express TGFβ receptors following cell activation (Friedman *et al.*, 1994). Autocrine expression of TGFβ augments HSC fibrogenesis. The sinusoidal endothelial cell also expresses a splice variant of fibronectin during early liver injury that has been shown to stimulate HSC activation (Jarnagin *et al.*, 1994).

2. Molecular mechanisms controlling collagen synthesis

2.1. *Type I collagen biosynthesis*

Type I collagen is the most abundant protein in the body. In the liver collagen types I, III, IV, V, VI, XIV (undulin), and XVIII (endostatin) have been found (Milani *et al.*, 1990; Milani *et al.*, 1994; Schuppan *et al.*, 1998). All collagen types share certain fundamental structural features. They are triple helical molecules in which each strand represents a helical polypeptide chain rich in proline and hydroxyproline. A glycine residue is present at every third position in the polypeptide chain. These strands, called α chains, aggregate to form a triple helical structure, which is sometimes interrupted in some collagen types by globular protein domains. The triple helices become cross-linked forming the classical collagen fibrils with each collagen type displaying unique physiochemical properties.

Type I collagen undergoes a complex biosynthetic pathway which allows for multiple levels for regulation (Fig. 1.2) (Vuorio and de Crombrugghe, 1990; Brenner *et al.*, 1994; McLaughlin and Bulleid, 1998; Brenner *et al.*, 2000; Kagan, 2000). Type I collagen is composed of two α1(I) chains and one α2(I) chain. Although located on different chromosomes, they are coordinately regulated in a tissue-specific, developmental, and inducible manner. Once transcribed the mRNAs are processed in the nucleus. The mature mRNA molecule is transported to the cytoplasm where it is translated into protein. Both α1(I) and α2(I) collagen mRNAs code for "pre-pro-peptide" chains. The

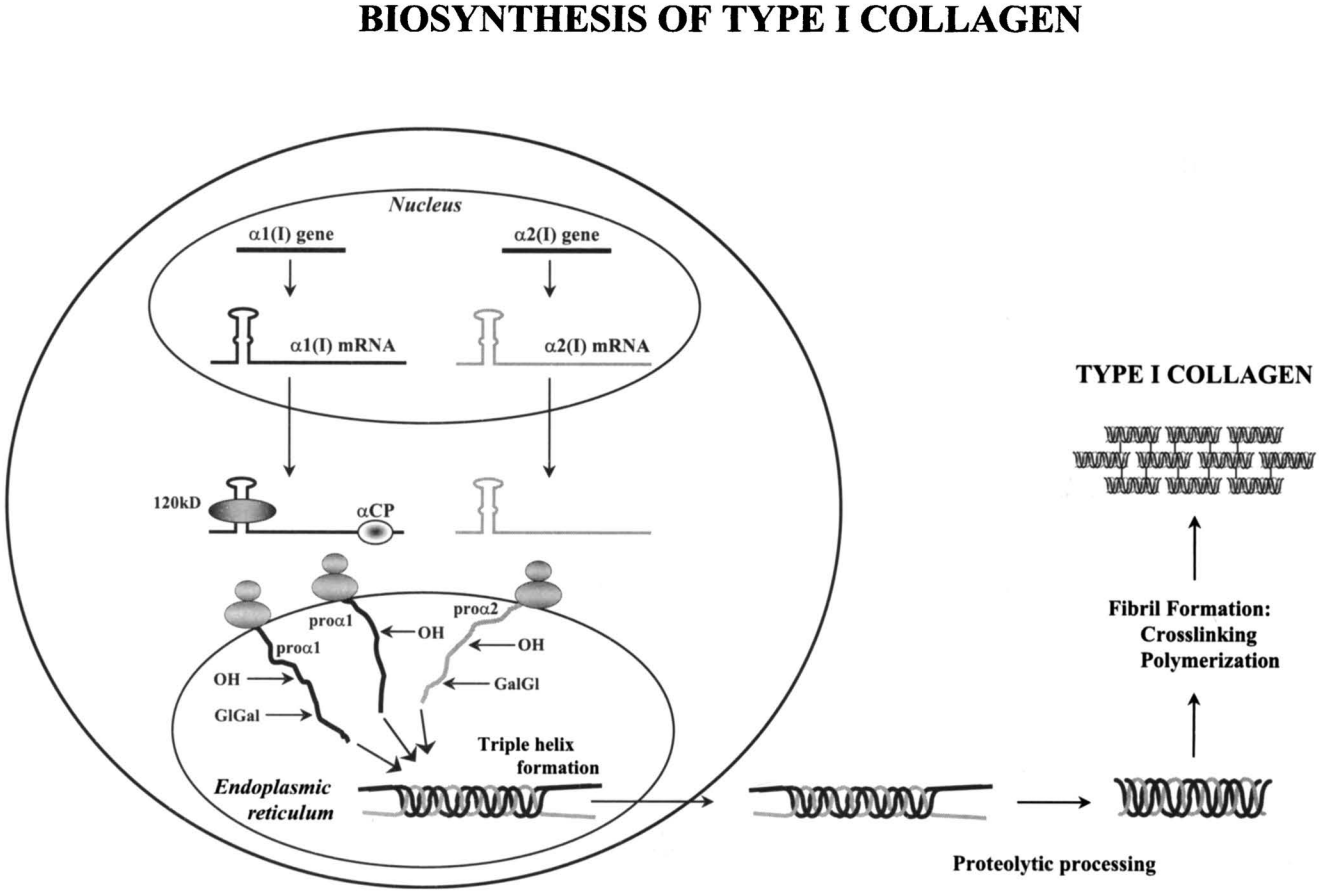

Fig. 1.2: Collagen Biosynthesis. Collagen synthesis involves a complex series of molecular steps involving mRNA stabilization, complex translational and posttranslational modifications, and transport from the cell. This complex process allows for multiple levels to regulate collagen expression.

presence of the "pre-peptide", or often called "leader" sequence directs the protein molecule to the endoplasmic reticulum. The "pro-peptide" chains contain both amino- and carboxy-terminal terminal protein sequences that are important for appropriate chain aggregation. Inter- and intrachain disulfide bond formation in these globular terminal regions guide α-chain aggregation. The propeptides, after removal, can also regulate collagen gene transcription by a poorly understood mechanism. The leader sequences are cleaved as the prepropeptide α chains enter the rough endoplasmic reticulum where important posttranslational modifications occur. Hydroxylation of some prolyl and lysyl residues to hydroxyproline and hydroxylysine is catalyzed by three hydroxylases, each requiring oxygen, α-ketoglutarate, ferrous iron, and ascorbate as cofactors (Kivirikko and Myllyharju, 1998). Within the triple helical region every lysine residue is hydroxylated while every third proline residue is hydroxylated. These reactions occur only when the prolyl or lysyl residue occupies a specific position in the α chain. These posttranslational modifications are required for α-chain interactions to form the triple helical procollagen molecule and to stabilize the formation of the triple helical structure (Kivirikko and Myllyharju, 1998). Some of the hydroxylated prolyl or lysl residues are also glycosylated (Brodsky and Ramshaw, 1997; McLaughlin and Bulleid, 1998). The procollagen molecule passes through the Golgi apparatus, in which the propeptides are cleaved when the protein is secreted into the extracellular space. This helps stabilize formation of the triple helix. Once the propeptides are cleaved, spontaneous aggregation of mature collagen molecules occurs, forming collagen fibrils. Intramolecular and intermolecular covalent cross-linking occurs which results in full tensile strength for the collagen fibril structure. These extracellular cross-linkages are enzyme-catalyzed reactions.

2.2. *Regulation of α1(I)collagen gene in HSC*

Increase in type I collagen expression in activated HSCs results from a 2–3-fold increase in the transcription rate coupled with a 16–20-fold increase in the stabilization of the α1(I) collagen mRNA (Stefanovic *et al.*, 1997). Therefore, both transcriptional and posttranscriptional regulatory processes control collagen gene expression in the activated HSC. To assess the minimal amount of genetic information needed for appropriate expression of the α1(I) collagen gene, a transgenic mouse was constructed that contained a single copy of the human α1(I) collagen gene which included the entire coding region along with 1.6 Kb of upstream sequence and 20 Kb of downstream sequence (Wu *et al.*, 1990). It was reported that transgene expression was appropriately expressed in all tissues as the endogenous gene. Initial control of gene expression is typically regulated by transcriptional mechanisms. This is true for collagen regulation in the HSC. Transcriptional regulation is usually controlled by proteins interacting with specific sites on the DNA. These regulatory sites are often located in the 5'-flanking region of a gene; however, transcriptional regulatory sites are found

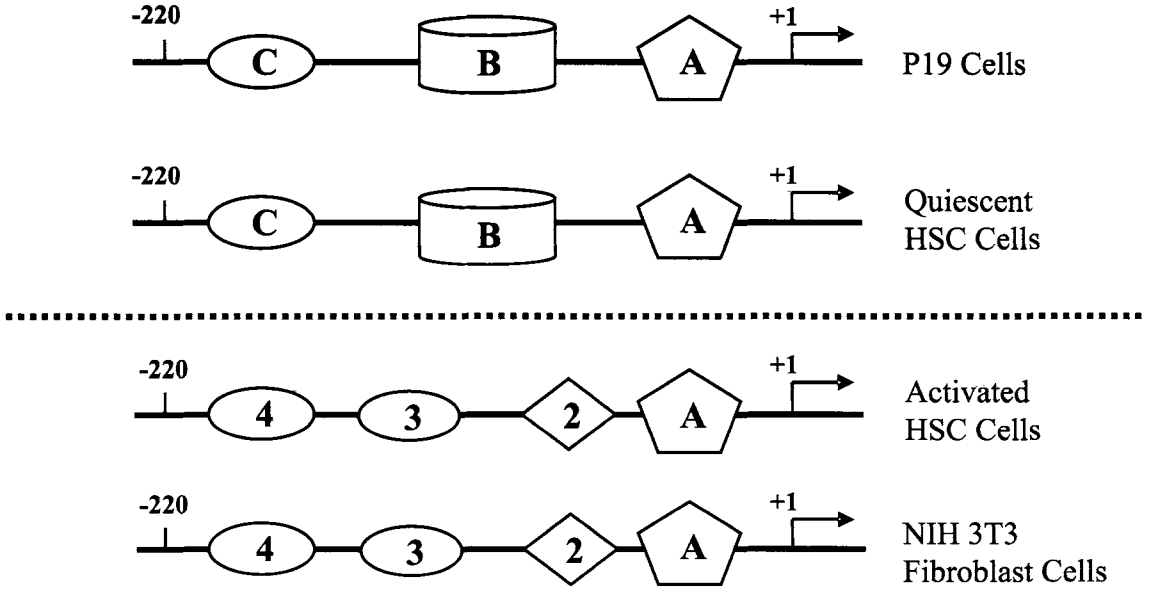

Fig. 1.3: DNA-Protein Interactions in Quiescent and Activated HSCs. The relative locations of DNA-protein interactions, identified by DNase I footprinting analysis, from quiescent, activated, collagen-producing cells (NIH 3T3 cells) and noncollagen-producing cells (P19 cells) are indicated. Interestingly, Noncollagen-producing cells and quiescent HSCs exhibit a similar footprint pattern while collagen-producing NIH 3T3 cells and activated HSCs show similar footprint patterns.

elsewhere, including far upstream of the transcriptional start site, in the 3'-flanking region of genes, and within intronic regions. Regulation of type I collagen gene expression has been more extensively studied for the α1(I) collagen gene than the α2(I) collagen gene. Several studies have identified regulatory elements within the 5'-flanking region, the promoter, the first intron, and the 3'-flanking region of the human, murine, and rat α1(I) collagen gene (Rossouw *et al.*, 1987; Rippe *et al.*, 1989; Rippe *et al.*, 1997). Recently, several DNase I hypersensitive sites were located as far upstream as 20 Kb from the α1(I) collagen gene promoter (Salimi-Tari *et al.*, 1997; Krempen *et al.*, 1999).

Several studies have identified transcriptional regulatory elements in the α1(I) collagen gene in the HSC. Transgenic mouse studies coupled with transient transfection of collagen reporter genes into activated HSCs have demonstrated that only 220 base pairs (bp) of the promoter region of the α1(I) collagen gene are required to direct high levels of expression of the α1(I) collagen gene in the liver (Brenner *et al.*, 1993, Houglum *et al.*, 1995, Rippe *et al.*, 1995). DNase I footprinting analysis, a technique used to locate regions of specific DNA-protein interactions, demonstrated marked changes in protein binding to the α1(I) collagen gene promoter following HSC activation (Fig. 1.3) (Rippe *et al.*, 1995). The pattern of protein binding obtained from quiescent HSC extracts was remarkably similar to the pattern obtained from other noncollagen producing cells (P19 cells). Three regions of DNA-protein interactions have been observed in the α1(I) collagen gene promoter; however, the identity of the proteins interacting with these sites has not been elucidated. Following HSC activation the pattern of protein binding to the promoter changes. Using nuclear extracts obtained from activated HSCs four sites wherein proteins specifically interact in the promoter region were identified, within 220 bp of the transcriptional start site (Rippe *et al.*, 1995). These sites are similar in position to those previously identified for the α1(I) collagen gene promoter using extracts obtained from fibroblast cells (Brenner *et al.*, 1989; Nehls *et al.*, 1991). These sites were identified as footprints 1–4 (FP1–FP4). In fibroblast cells FP1 and FP2 represent mutually exclusive overlapping binding sites for the transcription factors Sp1, a GC box binding protein, and NF-I, a CCAAT box binding protein (Fig. 1.4) (Nehls *et al.*, 1991). Both transcription factors were found to act as transcriptional activators of the α1(I) collagen gene promoter; however, Sp1 was a much more potent transcriptional activator compared to NF-I (Nehls *et al.*, 1992). In HSCs, two proteins were found to interact with FP1 and FP2. Sp1 was shown to interact with these sites; however, NF-I did not bind and the identity of the second transcription factor that interacts with FP1 and FP2 remains unknown. Contrarily, binding of the CCAAT binding factor CBF markedly increased following HSC activation and an oligonucleotide containing a consensus CBF binding site weakly competed for protein binding to FP2 in electrophoretic mobility shift assays (Rippe *et al.*, 1995). It is noteworthy that following HSC activation, binding activity of Sp1, the potent transcriptional activator for the α1(I) collagen gene, to the collagen gene promoter significantly increased (Rippe *et al.*, 1995).

HSC activation is also associated with induction of another transcription factor, a Kruppel-like factor, Zf9, which is induced *in vivo* during early hepatic fibrosis (Ratziu *et al.*, 1998). Zf9, like Sp1, is believed to regulate collagen gene expression in activated HSCs by binding to the GC motifs present in the α1(I) collagen gene promoter, as well as in the promoter regions of TGFβ1 and in the TGFβ type I and II receptor genes (Ratziu *et al.*, 1997; Kim *et al.*, 1998; Kohima *et al.*, 2000). In addition, Zf9 appears to regulate expression of the collagen-specific molecular chaperone HSP47, thereby assisting in regulation of type I collagen and promoting the fibrogenic nature of the activated HSC (Yasuda *et al.*, 2002).

Negative regulators of transcription were found to interact with both FP3 and FP4, initially identified as IF-1 and IF-2 respectively in fibroblast cell extracts and now accepted as binding sites for the transcriptional regulatory factor c-Krox (Fig. 1.4) (Karsenty and de Crombrugghe, 1990, Nehls *et al.*, 1991). It is not known whether IF-1 and IF-2 are responsible for interacting with FP3 and FP4 in HSCs.

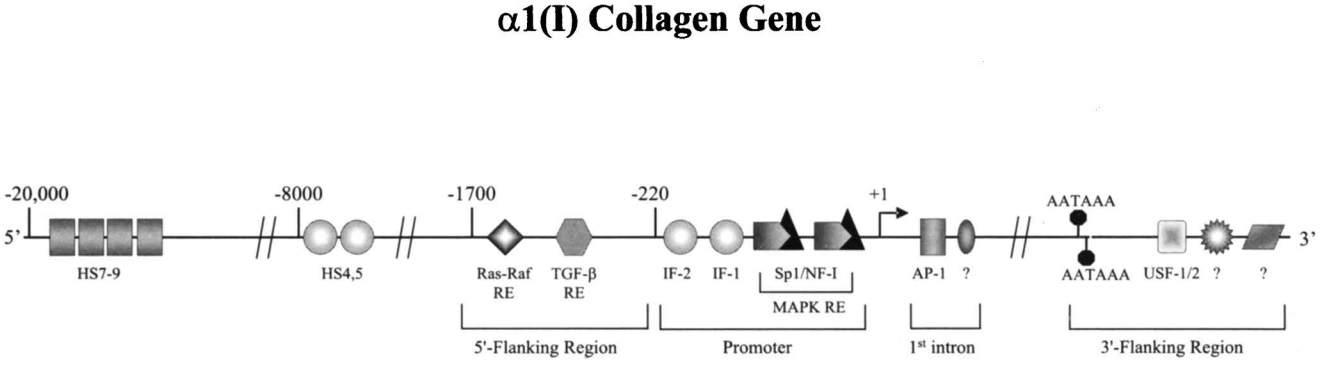

Fig. 1.4: Regulatory sites identified in the α1(I) collagen gene are indicated. Transcriptional regulatory sites have been reported in the far 5' flanking region, the proximal promoter, first intron, and in the 3' flanking region.

Interestingly, binding activity of the protein that interacts with FP4 is not altered following HSC activation.

Several lines of evidence have suggested that besides the proximal promoter region of the α1(I) collagen gene additional regulatory elements may be located at more distal sites in both the 5'- and 3'-flanking regions (Fig. 1.4). Analysis of the chromatin structure of the α2(I) collagen gene revealed several DNase I hypersensitive sites that provide transcriptional enhancer activity in transgenic mice (Bou-Gharios *et al.*, 1996). DNase I hypersensitive sites are believed to represent areas of open chromatin structure, where nucleosome binding does not occur, which then allows for transcription factors to access regulatory sites on the DNA. Analysis of the α1(I) collagen gene has also revealed the presence of DNase I hypersensitive regions in both the distal 5' and 3' regions that are at positions similar to those previously identified in the human α(I) collagen gene, thus supporting a conserved role of these potential regulatory sites between species (Barsh *et al.*, 1984; Ratziu *et al.*, 1998). Several of the DNase I hypersensitive sites located in the 5'-flanking region are only present in collagen-producing cells which strongly implicates a role of these sites in regulating collagen gene expression (Ratziu *et al.*, 1998). Recently, the DNase I hypersensitive sites located in the 5'-flanking region were investigated in HSCs. A total of 7 DNase I hypersensitive sites were identified in the 5'-flanking region. Two clusters of hypersensitive sites are present, one located between 7–8 Kb and the second between 15–20 Kb upstream of the transcriptional start site. The fact that these sites are present in collagen-producing cells, but not in non collagen-producing cells, implicates a role for these elements in collagen gene expression. Although these sites do not act as typical transcriptional enhancers, since they do not stimulate reporter gene activity in transient transfection assays, they do positively regulate collagen gene expression *in vivo* in transgenic mice (Krempen *et al.*, 1999; Yata *et al.*, 2003). It was recently shown that the DNase I hypersensitive sites located between 7–8 Kb upstream of the transcriptional start site of the α1(I) collagen gene enhanced collagen gene expression in HSCs and regulated the reporter gene activity, which contained approximately 1,600 bp of 5'-flanking sequence, in a manner similar to that of the endogenous α1(I) collagen gene in both culture activated HSCs and in the liver following a fibrogenic stimulus (Yata *et al.*, 2003).

The 3'-flanking region of the α1(I) collagen gene also contains regulatory elements that control expression of the α1(I) collagen gene promoter (Fig. 1.4). This region contains a DNase I hypersensitive site and has been included in gene fragments efficiently expressed in transgenic mice *in vivo* and in transfected fibroblasts in culture (Kahari *et al.*, 1990, Olsen *et al.*, 1991). Transgenic mice lacking the 3'-flanking region failed to provide high levels of transgene expression, while mice containing this region did (Sokolov *et al.*, 1995). Transient transfections in fibroblast cells using a luciferase reporter gene driven by the α1(I) collagen promoter and portions of the 3'-flanking region demonstrated the presence of transcriptional regulatory elements within the 3'-end of the gene (Rippe *et al.*, 1997). A binding site for upstream stimulatory factors 1 and 2 (USF1/2) was located and subsequently shown to stimulate transcription of the collagen gene promoter when positioned 3' of the luciferase reporter gene (Rippe *et al.*, 1997). In accordance with the regulatory role controlling α1(I) collagen gene expression, binding activity of USF-1/2 was found to increase following HSC activation (unpublished data).

A transcriptional regulatory role for the first intron in the α1(I) collagen gene has been a controversial issue. Both positive and negative regulatory elements have been located in the first intron (Fig. 1.4) (Bornstein *et al.*, 1987; Bornstein *et al.*, 1988; Bornstein and McKay, 1988; Rippe *et al.*, 1989). One study showed that AP1 binding in the first intron is involved in stimulating activity of the α1(I) collagen gene promoter (Liska *et al.*, 1990). However, another study showed that reporter genes containing segments of the first intron do not augment gene expression in transient transfection assays (Rippe *et al.*, 1989). A transcriptional enhancer has also been reported to be located within the first intron of the α1(I) collagen gene (Bornstein *et al.*, 1987; Roussouw *et al.*, 1987). However, studies using transgenic knockout mice have shown that this region of the gene is not essential for development and thus question the biological importance of this putative enhancer element (Slack *et al.*, 1991; Sokolov *et al.*, 1995). Therefore, the role of the first intron in regulating α1(I) collagen gene expression remains controversial.

Several signal transduction pathways have been shown to stimulate extracellular matrix protein production in HSCs. For HSCs, PDGF and TGFβ represent the major mitogens that stimulate HSC proliferation and fibrogenesis respectively. Both cytokines have been shown to stimulate the Ras-Raf-MAPK signaling pathway (Marshall, 1995). When dominant negative forms of Ras or Raf were introduced into HSCs, an increase in α1(I) collagen reporter genes was observed; however, when MAPK signaling was blocked, using a dominant negative form of ERK, a decrease in α1(I) collagen gene expression was found (Davis *et al.*, 1996). The MAPK responsiveness was mapped to the Sp1 and NF-I sites in the proximal promoter region (Lang and Brenner, 1999). The Ras-Raf responsive element was mapped to an upstream region located approximately 1700 bp from the transcriptional start site where a novel 60 kDa DNA binding protein expressed by activated HSCs was bound (Davis *et al.*, 1996).

The phosphatidylinositol-3-kinase (PI3-K) signaling pathway has recently been shown to influence type I collagen expression in activated HSC (Fig. 1.5). Following PDGF binding to its receptor on the cell surface, activation of the focal adhesion kinase (FAK)—PI3-K–Akt signal pathway occurs. This signaling pathway is traditionally linked to cell proliferation in many cell types, including the HSC (Reif *et al.*, 2003). Interestingly, inhibiting PI3-K activity reduced both steady state levels of α1(I) collagen mRNA and blocked secretion of type I collagen. Therefore, this study identified PI3-K as an important signaling molecule for the development of fibrosis because it is directly involved in two critical aspects

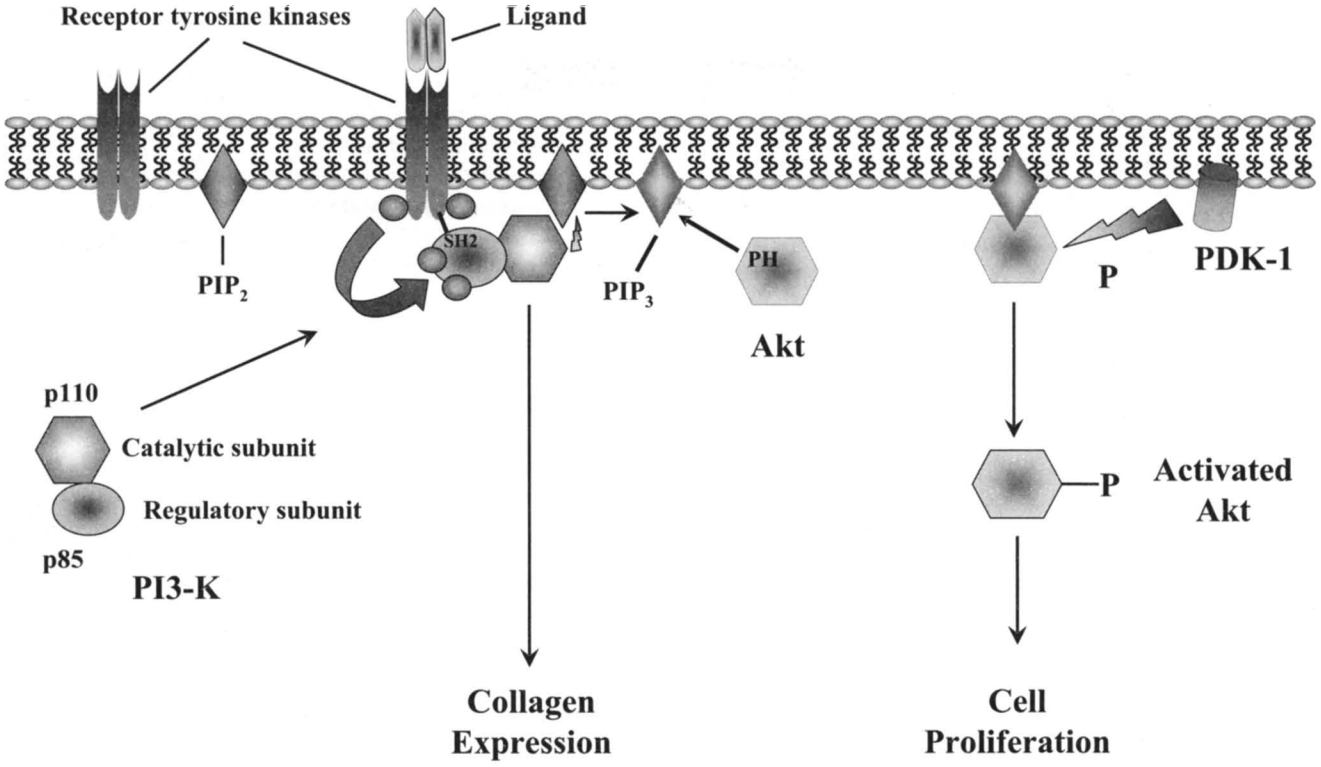

Fig. 1.5: Phosphatidylinositol 3-kinase signaling in the HSC. Following activation of a receptor tyrosine kinase by its ligand (i.e. PDGF binding to the PDGF receptor) PI3-K is directed to the activated receptor by its SH-2 domain (SH). This association activates PI3-K, which subsequently phosphorylates phosphatidyl inositols in the cell membrane at the D-3 position (PIP_2 to PIP_3). Akt is then attracted to the cell membrane by PIP_3s via the plekstrin homology domain (PH). Once located at the cell membrane Akt becomes activated by a phosphorylation event by PDK-1. Once activated Akt leads to HSC proliferation. Activated PI3-K also controls type I collagen gene expression.

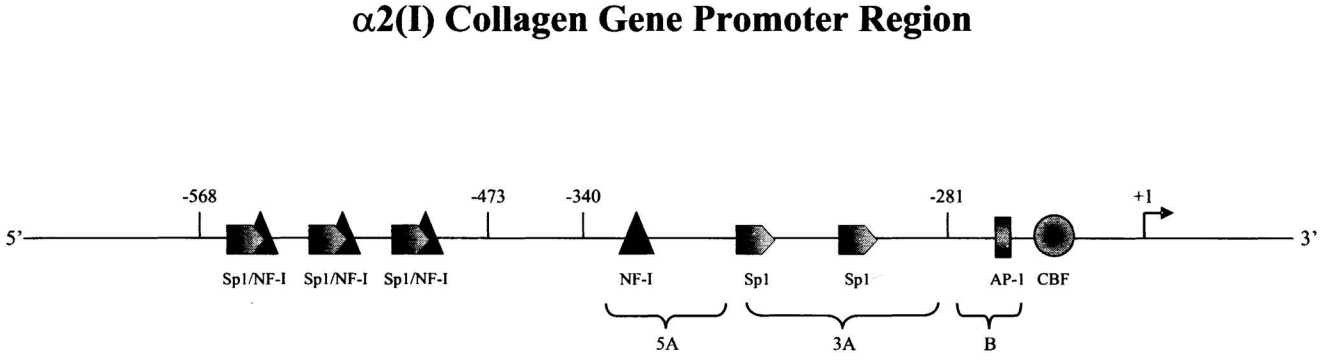

Fig. 1.6: Known transcriptional regulatory sites identified in the α2(I) collagen gene proximal promoter region are indicated.

of HSC fibrogenesis, namely HSC proliferation and collagen gene expression (Reif *et al.*, 2003).

2.3. *Alpha2(I) collagen gene regulation in HSCs*

Transcriptional analysis of the α2(I) collagen gene in HSCs has been less studied than the α1(I) collagen gene. Although the α1(I) and α2(I) collagen genes are coordinately expressed, the transcriptional regulatory elements of these two genes are differently organized. A major regulatory region, driving high levels of activity, is located within 350 bp of the transcriptional start site in the murine α2(I) collagen (Fig. 1.6) (Goldberg *et al.*, 1992). This region is GC rich and contains a TGFβ responsive element located between -330

to -255 (Inagaki *et al.*, 1994; Inagaki *et al.*, 1995). Two sites of DNA-protein interactions are located within this region, called the A box, which is divided into two segments, 5A and 3A, and the B box (Inagaki *et al.*, 1994). The precise location of the TGFβ responsive element is not certainly known but it has been suggested that this response element maps to an AP1 recognition sequence overlapping the 3' portion of the B box (Chung *et al.*, 1996). On the other hand, the TGFβ responsive element has also been suggested to be located within boxes 3A and B where Sp1 was found to mediate TGFβ responsiveness of the α2(I) collagen gene (Greenwel *et al.*, 1997, Inagaki *et al.*, 1994). Like the α1(I) collagen gene promoter, the CCAAT motif binding factor, CBF, binds to a CCAAT box located in the proximal promoter region (Oikarinen *et al.*, 1987). An inhibitory factor, IF-1, interacts with the promoter region between –165 to –155 and NF-I binds to a region located between –315 to –295 (Karsenty and de Crombrugghe, 1991; Anania *et al.*, 1995). Another region, located between –554 to –473, contains three GC rich elements that bind the transcription factors Sp1 and NF-I from proteins isolated from activated HSCs (Miao *et al.*, 1997). Binding activity to these upstream regions appears to be necessary for optimal activity of the α2(I) collagen gene. In addition to these transcriptional regulatory sites, several DNase I hypersensitive sites have been located far upstream in the α2(I) collagen gene 5'-flanking region; their role in regulating expression of the α2(I) collagen gene in HSC has yet to be investigated however (Bou-Gharios *et al.*, 1996; Antoniv *et al.*, 2001).

3. Mediators influencing collagen expression in HSCs

Several physiological mediators have been identified that directly influence collagen gene expression in the HSC (Fig. 1.7). These mediators can either increase or decrease collagen gene expression. Important mediators of collagen gene expression during liver fibrosis and what is currently known about the molecular mechanisms by which these mediators influence collagen gene expression in the HSC, are briefly summarized below.

3.1. Oxidative stress and collagen gene expression (see Chapter 7 by Zamara et al.)

Oxidative stress is an important factor in the development and progression of liver fibrosis caused by different etiologies including alcohol ingestion, viral infection, iron or copper overload, cholestasis, or hepatic blood congestion. Oxidative stress is a condition wherein the generation of highly reactive oxygen intermediate species (ROIs) occurs. The ROIs can oxidize many proteins, but lipids are a major target. Oxidized molecules often have detrimental effects on cellular metabolism. The role of oxidation of lipids, lipid peroxidation, has been extensively studied in liver fibrosis.

Mediators Influencing Collagen Gene Expression

- **Oxidative Stress**
  - **Reactive oxygen intermediates (ROIs)**
  - **Lipid peroxidation products**
    - **4-hydroxy-2,3-nonenal (HNE)**
    - **malondialdehyde (MDA)**
- **Ethanol**
  - **Acetaldehyde**
  - **Reactive oxygen intermediates (ROIs)**
- **Iron**
- **Copper**
- **TGFβ**
- **TNFα**
- **Interferon-α**
- **Interferon-γ**
- **Leptin**

Fig. 1.7: Mediators influencing collagen gene expression in the HSC. Several mediators have been identified that influence collagen gene expression in the HSC. These either increase or decrease collagen gene expression. The molecular mechanisms by which these mediators influence collagen expression may involve similar pathways (see text).

Lipid peroxidation is associated with tissue injury and fibrogenesis of several pathologic disorders, including atherosclerosis, iron overload, and porphyria, as well as with ethanol, bleomycin, and CCl_4-induced liver toxicity. ROIs induce formation of lipid peroxidation products from polyunsaturated fatty acids of phospholipids present within the cell membrane. These are highly susceptible to attack by free radicals, and subsequent reactions with oxygen generate lipid peroxides. Lipid peroxides may be further oxidized to aldehydes such as 4-hydroxy-2,3-nonenal (HNE) and malondialdehyde (MDA). Both HNE and MDA levels and their modified protein adducts are increased in alcoholic patients accompanied by increased collagen deposition (Niemela *et al.*, 2000; Rolla *et al.*, 2000). In cultured fibroblast cells these highly reactive aldehydes stimulate collagen gene expression. Incubating cells with exogenous MDA induce an increase in collagen gene expression (Chojkier *et al.*, 1989; Geesin *et al.*, 1991). Furthermore, decreasing the basal level of lipid peroxidation in these cultures with α-tocopherol (vitamin E) prevents lipid peroxidation and blocks stimulation of collagen production and collagen gene expression by ascorbic acid. Treatment with methylene blue has an inhibitory effect on collagen induction similar to products of lipid peroxidation. In HSCs treatment with either HNE or MDA strongly stimulates type I collagen gene expression (Parola *et al.*, 1993; Parola *et al.*, 1996; Poli and Parola, 1997; Garcia-Ruiz *et al.*, 2002). MDA stimulated collagen production was shown to involve enhanced Sp1 and Sp3 synthesis and associated binding to the GC boxes

located in the proximal promoter region of the α1(I) collagen gene promoter (Garcia-Ruiz *et al.*, 2002).

3.2. *Ethanol mediated collagen expression*

Excess ethanol consumption represents the number one cause of liver fibrosis in the United States. Development of oxidative stress is strongly associated with ethanol-induced liver fibrosis. The fibrogenic effect of ethanol is due in part to the generation of ROIs (including 1-hydroxyethyl) via cytochrome CYP2E1 (Knecht *et al.*, 1990). Inhibiting CYP2E1 reduces ROI formation and generation of lipid peroxidation products in ethanol-fed animals (Morimoto *et al.*, 1995; Albano *et al.*, 1996). ROIs generated from ethanol metabolism can be produced by NADPH oxidase present in Kupffer cells (Kono *et al.*, 2000). The generated ROIs are believed to directly affect HSCs, resulting in increased ECM production (see above). In addition, ROIs can activate Kupffer cells increasing production of both proinflammatory and profibrogenic cytokines.

Ethanol also promotes fibrogenesis through its primary metabolite, acetaldehyde. The mechanism by which acetaldehyde increases collagen transcription is not known. Acetaldehyde covalently binds to proteins via Schiff bases, especially with the α-amino group of lysines, forming acetaldehyde-protein adducts. These adducts can be detected in the liver of ethanol-fed rats, and antibodies to acetaldehyde adducts are present in the serum of most alcoholic individuals (Holstege *et al.*, 1994; Lieber, 1997; Viitala *et al.*, 1997; Niemela, 1999b; Niemela *et al.*, 2000; Rolla *et al.*, 2000). Perhaps formation of adducts of acetaldehyde with proteins involved in transcriptional regulation is the mechanism by which collagen gene transcription increases. Acetaldehyde is a highly reactive compound that can form aldehyde adducts with many proteins that often alters their function. Acetaldehyde stimulates collagen expression in fibroblasts and in cultured HSCs in a protein synthesis dependent manner (Pares *et al.*, 1994; Anania *et al.*, 1995; Miao *et al.*, 1997). This stimulatory effect on collagen gene expression may be due to acetaldehyde increasing binding of transcription factors to promoters of the collagen genes (Pares *et al.*, 1994; Anania *et al.*, 1995; Miao *et al.*, 1997). Treatment of HSCs with acetaldehyde increases protein binding of the transcription factor BTEB to a GC rich region located between –1484 and –1476 in the α1(I) collagen gene promoter (Chen and Davis, 2000). Activation of c-jun nuclear kinase (JNK) appears to be involved in mediating the increase in collagen expression by acetaldehyde by increasing transcription factor binding to the collagen promoter (Chen and Davis, 2000; Anania *et al.*, 2001). Inhibiting JNK activity decreased BTEB protein levels and reduced collagen gene expression (Chen and Davis, 2000). In another study treatment of HSCs with acetaldehyde increased protein levels and binding activity of the transcription factor C/EBPβ to a site located between –365 and –335 in the α1(I) collagen gene promoter, apparently mediated by a H_2O_2 dependent mechanism (Attard *et al.*, 2000; Greenwel *et al.*, 2000). Mutational analysis of this site prevented acetaldehyde-induced collagen expression in the HSC. Activation of collagen gene expression following acetaldehyde treatment appears to involve calcium independent activation of protein kinase C (PKC) (Casini *et al.*, 1994; Anania *et al.*, 1999). Inhibitors of PKC activity were found to decrease both α1(I) collagen and α2(I) gene expression in HSCs treated with acetaldehyde.

3.3. *Role of iron and copper overload in collagen gene expression*

Iron or copper overload, as seen in hemochromatosis and Wilson's disease respectively, is another source for ROI formation and development of fibrosis. The accumulation of iron results in formation of reactive oxygen through the Fenton reaction. This leads to oxidation of internal membrane lipids of the mitochondria, microsomes, and lysosomes, resulting in generation of lipid peroxidation products (HNE and MDA) and associated protein adduct formation, causing cell injury due to functional problems with these cellular structures (Bacon and Britton, 1990; Britton *et al.*, 1990). Increased levels of iron can induce collagen gene expression and HSC proliferation (Gualdi *et al.*, 1994; Pietrangelo *et al.*, 1994). The role of oxidative stress in the development of iron-induced fibrosis is supported by studies wherein antioxidants, such as Vitamin E or silybin, were used to reduce levels of lipid peroxidation products and the development of fibrosis (Pietrangelo *et al.*, 1995a, Pietrangelo, 1995b). However, another study showed that lipid peroxidation products were not responsible for the increase in collagen expression following iron treatment in HSCs (Cardi *et al.*, 2002). Increased lipid peroxidation caused by iron overload can activate Kupffer cells which can subsequently induce ROI formation and begin to express fibrogenic cytokines that stimulate HSC production of excess ECM components. The molecular mechanisms responsible for iron-induced collagen gene expression are not known; however, increased binding of Sp1 to the collagen gene promoter has been shown in HSCs following iron treatment (Ruiz *et al.*, 2000).

More work has been done to investigate the mechanisms responsible for iron overload in causing fibrosis than for copper overload; however, copper probably mediates a similar induction of ROI formation. Copper overload results in a chronic inflammatory response accompanied by significant fibrosis, which can eventually lead to development of cellular responses just as iron does, with generation of excess ROIs eventually leading to liver cirrhosis. It has been shown that excess copper can increase products of lipid peroxidation, which can be reduced with antioxidant (Vitamin E) treatment (Solol *et al.*, 1990).

3.4. *Regulation of type I collagen by TGFβ*

However, the transforming growth factor beta (TGFβ) is the most potent fibrogenic cytokine known for the HSC. TGFβ administered exogenously induces pulmonary, kidney, and

liver fibrosis (Kanzler *et al.*, 1999). Increased TGFβ expression is also found in experimental models of hepatic fibrosis, including fibrosis induced by bile duct ligation, CCl_4 administration, and schistosomiasis infection (Czaja *et al.*, 1989; Parola *et al.*, 1992; Bissell *et al.*, 1995; De Bleser *et al.*, 1997). Increased TGFβ mRNA levels are found in patients with alcohol-induced and viral-induced cirrhosis that correlate with the extent of liver fibrosis (Castilla *et al.*, 1991; Annoni *et al.*, 1992; Bachem *et al.*, 1992). Studies using transgenic mice have provided strong evidence for a fibrogenic role of TGFβ in the development of liver fibrosis. A constitutively expressing form of active TGFβ caused increased levels of TGFβ that were associated with increased type I collagen deposition and development of liver fibrosis (Sanderson *et al.*, 1995; Clouthier *et al.*, 1997; Kanzler *et al.*, 1999).

TGFβ belongs to a superfamily of cytokines that influences cell growth and tissue patterning in many tissue types. TGFβ superfamily members regulate transcription of several genes including those involved with cell cycle control, cell adhesion molecules, homeobox genes, and extracellular matrix proteins. TGFβ represents a group of five separate peptide hormones (TGFβ1-5) primarily expressed by inflammatory cells and platelets. However, the HSC has also been shown to express TGFβ. TGFβ family members play important roles in several physiological processes including embryogenesis, wound repair, inflammation, carcinogenesis, immunosuppression, and fibrogenesis. A direct fibrogenic role for TGFβ was shown when direct exogenous administration of TGFβ induced fibrosis in the lung, kidney, and liver and, therefore, TGFβ is considered a potent fibrogenic cytokine. This holds true for the HSC as well (Kanzler *et al.*, 1999). During experimental models of hepatic fibrosis, including bile duct ligation, CCl_4 administration, and schistosomiasis infection, a prolonged increase in TGFβ expression was observed (Bissell *et al.*, 1995; De Bleser *et al.*, 1997). Patients with alcohol-induced and viral-induced cirrhosis also exhibit increased TGFβ mRNA levels.

TGFβ promotes fibrogenesis by multiple mechanisms. First, TGFβ can induce expression and decrease degradation of extracellular matrix proteins, thereby disrupting the delicate balance of ECM homeostasis. This results in an overall net increase in deposition of ECM proteins. An increase in TGFβ receptor expression occurs following HSC activation that may allow for an auto-stimulatory loop enhancing the TGFβ effects in HSCs. An increase in rate of HSC activation occurs following TGFβ treatment (Friedman and Arthur, 1989). TGFβ increases expression of extracellular matrix proteins, such as type I collagen and fibronectin. Type I collagen synthesis increased in cultured HSCs following TGFβ treatment (Matsuoka *et al.*, 1989; Gong *et al.*, 1998). This increase is due to an enhanced transcription rate and posttranscriptional regulation. Three potential TGFβ responsive elements have been identified in the α1(I) collagen gene; however, a detailed characterization of the transcription factors regulating TGFβ-responsiveness for the α1(I) collagen gene has not been performed. One TGFβ responsive element was located approximately 1,600 bp upstream of the transcriptional start site in the rat α1(I) gene while a second element was located to the 3' flanking region of the murine α1(I) collagen gene (Ritzenthaler *et al.*, 1991; Brenner *et al.*, 1994). A third putative TGFβ responsive element was mapped between -370 and -344, where TGFβ treatment of cells induced binding of a protein complex containing the transcription factor C/EBPβ with H_2O_2 acting as a second messenger (Garcia-Trevijano *et al.*, 1999). TGFβ can decrease expression of Sp3, considered to be a negative regulator of transcription, thereby allowing for increased binding of the potent transcriptional activator Sp1, to the α1(I) collagen gene promoter (Nehls *et al.*, 1991; Galera *et al.*, 1994). Characterization of the TGFβ responsive elements in the α2(I) collagen gene have achieved greater attention.

Although the molecular mechanisms responsible for TGFβ-mediated enhanced expression of the α2(I) collagen gene have been more intensively investigated than the α1(I) collagen gene, the mechanisms underlying TGFβ-mediated responsiveness remain controversial. One mechanism is believed to involve AP1 binding to a region overlapping the 3' end of Box B, mapped between -265 and -241 in the α2(I) collagen gene promoter (Chung *et al.*, 1996). It is thought that a change occurs in the binding partners of AP1 with a transition from c-fos to Jun-B; partially supported by the fact that TGFβ induces *jun-B* gene expression, and antisense *jun-B* RNA reduced TGFβ stimulation of the α2(I) collagen gene promoter (Chang and Goldberg, 1995; Chung *et al.*, 1996). Another proposed mechanism for TGFβ induction of the α2(I) collagen gene involves Sp1 interacting with Boxes 3A and B in which a TGFβ-induced protein complex binds, of which Sp1 appears to be one of the protein members of this complex (Inagaki *et al.*, 1994; Greenwel *et al.*, 1997). Modulation of this TGFβ-induced complex by posttranslational modification, specifically involving tyrosine dephosphorylation modification, possibly of an Sp1 cofactor, that results in increased binding activity of the complex to the DNA (Greenwel *et al.*, 1995).

Intracellular signaling for TGFβ in HSCs is mediated in part by Smad proteins, in particular by Smad 2, 3, and 4 (Heldin *et al.*, 1997; Kawabata and Miyazono, 1999). Smad 3 is not required for HSC activation; however, it is required for maximal expression of α1(I) collagen gene expression in HSCs (Schnabl *et al.*, 2001). The molecular mechanism by which Smad 3 regulates α1(I) collagen gene expression is not known. In addition, TGFβ signaling also activates p38 MAPK signaling, leading to increased collagen gene expression (Varela-Rey *et al.*, 2002). Treatment of HSCs with TGFβ was shown to increase p38 activation and blocking p38 activity inhibited the increase in collagen expression following TGFβ treatment. Another study showed that TGFβ induced collagen gene expression in HSCs by an H_2O_2 dependent mechanism that involved binding of C/EBPβ to an element located between -370 and -344 in the α1(I) collagen gene promoter (Garcia-Trevijano *et al.*, 1999).

3.5. *TNFα mediated collagen regulation*

TNFα has been shown to inhibit type I collagen gene expression in cultured fibroblasts, HSCs, and in animals (Solis-Herruzo *et al.*, 1988; Buck *et al.*, 1996; Hernandez-Munoz *et al.*, 1997; Rippe *et al.*, 1999). TNFα has been shown to mediate its inhibitory effect on α1(I) collagen gene expression through a pertussis toxin sensitive G protein (Hernandez-Munoz *et al.*, 1997). Several studies have shown that the inhibitory effect of TNFα on collagen gene expression is mediated by regulatory elements present within the proximal promoter region (Hatamochi *et al.*, 1994; Mori *et al.*, 1996; Houglum *et al.*, 1998). However, the location of the TNFα inhibitory element is not without controversy. One study reported that TNFα inhibited binding of Sp1 to a GC rich region located in the 5' untranslated region of the first exon and that this inhibitory action is responsible for the inhibitory effects of TNFα on collagen gene expression. In another study TNFα was shown to inhibit α1(I) collagen by a p20c/EBPβ and C/EBPδ dependent mechanism that mapped between -378 and -345 in the proximal promoter region (Iraburu *et al.*, 2000). TNFα strongly activates the transcription factor NFκB in several cell types, including the HSC. Expression of the NFκB subunits p50 and p65 were shown to strongly inhibit expression of the α1(I) collagen gene promoter in both fibroblast cells and in HSCs. This inhibitory effect mapped to the proximal promoter region of the α1(I) collagen gene. Interestingly, it was found that the p65 NFκB subunit physically interacted with Sp1.

3.6. *Other mediators influencing collagen gene regulation*

Several additional mediators are known to influence collagen gene expression and the fibrogenic response of HSCs. These include interferon-α and -γ and more recently leptin. Interferon-γ treatment has been shown to inhibit collagen expression *in vitro* in several systems, including HSCs (Kahari *et al.*, 1990; Rockey *et al.*, 1992; Mallat *et al.*, 1995). The IFN-γ inhibitory element was located in a region surrounding –160 in the α2(I) collagen gene promoter (Higashi *et al.*, 1998). It was subsequently found that IFN-γ activated Jak1 and Stat1 signaling cascade which induced the TGFβ inhibitory protein Smad 7, which is believed to lead to interaction with p300/CBP transcriptional cofactors, resulting in suppression of Smad3/p300 stimulated α2(I) collagen gene transcription (Ghosh *et al.*, 2001).

Interferon-α treatment has been used to treat Hepatitis C viral infections with moderate results. Improvement in serum markers of liver fibrosis has been observed in some patients and the degree of liver fibrosis has improved following IFN-α treatment (Manabe *et al.*, 1993; Hiramatsu *et al.*, 1995; Suou *et al.*, 1995; Duchatelle *et al.*, 1998; Tsushima *et al.*, 1999). Together, these results suggest that IFN-α treatment may inhibit collagen gene expression. Recently, IFN-α treatment to transgenic mice harboring an α2(I) collagen reporter gene demonstrated a significant reduction in promoter activity following experimentally induced liver fibrosis. The inhibitory action was found to inhibit Smad3 stimulated transcription mediated by Stat1/p300 interaction (Inagaki *et al.*, 2003).

Recently, the peptide hormone leptin has gained a great deal of attention for its potential role in the development of liver fibrosis. In leptin-deficient mice (*ob/ob* mice) liver fibrosis was significantly reduced following stimulation of fibrosis with either CCl_4 or infection with the parasite *Schistosoma mansoni* (Potter and Mezey, 2002; Saxena *et al.*, 2002). *In vitro*, leptin has been shown to increase α2(I) collagen mRNA and protein levels and stimulate expression of the α2(I) collagen gene promoter in culture activated HSCs, but not in quiescent HSCs (Potter *et al.*, 1998; Saxena *et al.*, 2002; Tang *et al.*, 2002). In HSCs, it appears that leptin may mediate its profibrogenic response by augmenting TGFβ II receptor expression (Tang *et al.*, 2002).

4. Posttranscriptional regulation of type I collagen expression

There is compelling evidence that the steady-state level of many proteins is regulated at multiple steps. Especially when there is a large change in the amount of either mRNA or protein, it is likely that multiple steps in the metabolism of the mRNA and protein have been altered (Lindquist *et al.*, 2000). The combination of altering the rates or efficiency of multiple steps in metabolism of a protein results in the large changes observed. In the case of collagen type I, our recent work has shown that there is regulation of multiple steps resulting in a close to 70-fold increase in rate of synthesis of collagen type I as a result of activation of hepatic stellate cells (HSCs) (Stefanovic *et al.*, 1997). The steady-state level of α1(I) and α2(I) collagen mRNA is determined by the rate of transcription, the percentage of transcripts ultimately processed and transported to the cytoplasm, and the halflife of the mRNA in the cytoplasm. The amount of procollagen protein secreted out of HSCs is influenced not only by the amount of α1(I) and α2(I) collagen mRNAs present in the cytoplasm, but also by the rate of translation of the mRNAs (Stefanovic and Brenner, 2003), folding of nascent procollagen chains into a triple helix, and stability of the triple helix (Baker *et al.*, 1989). The molecular details of some of many of these posttranscriptional regulatory events are currently being elucidated. Here we review the regulation in synthesis of type I collagen with emphasis on regulation of processing, stability, and translation of α1(I) collagen mRNA.

4.1. *Regulation of α1(I) collagen mRNA processing*

Processing of mRNA includes removal of introns, attachment of 7 mG cap at the 5' end of an mRNA and cleavage and polyadenylation at the 3' end. Pre-mRNAs undergo processing cotranscriptionally. Capping enzymes, splicing factors, and cleavage and polyadenylation factors associate at the onset of transcription with the C-terminal tail of RNA polymerase II

(Hirose *et al.*, 1999). During transcription elongation these factors recognize signals on the nascent pre-mRNA and transfer to the pre-mRNA to initiate processing (Jurica and Moore, 2003; Misteli, 2000). Unprocessed mRNAs are degraded and there are several examples of how such quality control prevents accumulation of aberrant procollagen type I (Willing *et al.*, 1993; Willing *et al.*, 1994). For example, in one case of osteogenesis imperfecta (OI) type I a G $\rightarrow$ A mutation occurred at the donor site of intron 26. This mRNA retained intron 26, which introduced a premature stop codon. The mRNA did not accumulate in the cytoplasm, resulting in no truncated protein being made and a mild form of OI (Stover *et al.*, 1993). Several other cases have been reported wherein mutations introduced premature stop codons in α1(I) collagen mRNA. In all these cases the mRNA did not accumulate in the cytoplasm and in all cases OI was of a mild phenotype (Wenstrup *et al.*, 1990; Redford-Badwal *et al.*, 1996). The mechanism that prevents truncated proteins from being synthesized by unprocessed or mutated mRNAs is called nonsense mediated mRNA decay (NMD) (Maquat and Serin, 2001; Lejeune *et al.*, 2003) and is an important regulatory mechanism precluding the dominant phenotype of the mutations (Hentze and Kulozik, 1999; Noensie and Dietz, 2001).

Introns are not necessarily removed in 5' to 3' order. As pre-mRNA is synthesized, some introns are removed rapidly and some are processed slowly. In α1(I) collagen pre-mRNA removal of introns 5, 6, and 9 is rapid. Removal of intron 8 precedes removal of intron 7, both slow removing introns (Schwarze *et al.*, 1999). The mechanism that determines the rate of intron removal is completely unknown but has profound effects on the phenotype found in OI. At the intron 8 splice-donor site of the α1(I) collagen, a G+1 $\rightarrow$ A transition was identified that resulted in the production of several splice products from the mutant allele. These included one in which the upstream exon 7 was extended by 96 nt, others in which either intron 8 or introns 7 and 8 were retained, one in which exon 8 was skipped, and one that used a cryptic donor site in exon 8. The proportion of abnormal products suggested that exon 7 redefinition, intron 7 plus intron 8 inclusion, and exon 8 skipping all represented products of the impaired rapid pathway, whereas the intron 8 inclusion product resulted from use of the slow pathway. These results (Schwarze *et al.*, 1999) suggest that α1(I) collagen mRNA is spliced by two pathways, rapid and slow, and that utilization of the particular pathway determines the severity of the phenotype of OI. Utilization of the splicing pathway that results in accumulation of α1(I) collagen mRNA with a retained intron usually has a mild phenotype due to NMD, while skipping of an exon usually has a severe phenotype due to production of a truncated α1(I) collagen chain. What regulates utilization of the slow or rapid splicing pathways of α1(I) collagen pre-mRNA is completely unknown.

RNA tracks are localized accumulation of detached transcripts that extend beyond and to one side of the genes (Rosbash and Singer, 1993). They represent an early step of mRNA export from the nucleus and not merely a "Christmas tree" of nascent transcripts on the gene. For the α1(I) collagen gene such tracks have been observed to extend from the gene into regions of nucleoplasm called SC-35 domains (Johnson *et al.*, 2000). SC-35 domains are 20–40 large globular domains within a nucleus from 0.5–3 μM in size. They are enriched in splicing factor SC-35 as well as in other splicing factors (Shopland *et al.*, 2003). Since α1(I) collagen mRNA accumulates at one side of the gene and in an SC-35 domain, this indicates a slow or rate limiting step in the export of this mRNA 1–3 μM from the gene. Upon leaving the SC-35 domain the track of α1(I) collagen mRNA becomes less visible, suggesting rapid diffusion of the mRNA once it has passed through this domain (Johnson *et al.*, 2000). In one case of OI, a G $\rightarrow$ A mutation in the donor site of intron 26 caused retention of this intron in mRNA (Stover *et al.*, 1993). The mutant mRNA also showed tracks on one side of the gene that extended to an SC-35 domain. However, these tracks never left the SC-35 domain, suggesting that unprocessed α1(I) collagen mRNA is retained within the SC-35 domain (Johnson *et al.*, 2000). Retention ultimately leads to degradation of this aberrant mRNA and it never shows up in the cytoplasm. The result is a mild form of OI due to haploinsufficiency. Thus, there is a control checkpoint for α1(I) collagen mRNA, probably involving splicing, that is executed 1–3 μM from the gene in an SC-35 domain. The molecular mechanism of such quality control is not known but it may not differ from nonsense-mediated mRNA decay (NMD), which operates after export of mRNAs into the cytoplasm.

α1(I) collagen mRNA has two polyadenylation signals (AAUAAA) in the 3'UTR that are about 1 kb apart (Vuorio and de Crombrugghe, 1990). In all tissues both signals are utilized simultaneously, resulting in accumulation of two α1(I) collagen mRNAs of 4.5 Kb and 5.5 Kb in size. However, the relative amount of these mRNA species changes under various conditions. Besides the two AAUAAA signals, the sequences preceding these signals are 86% and 71% identical between human and mouse α1(I) collagen genes respectively (Natalizio *et al.*, 2002). A high degree of evolutionary conservation of noncoding sequence implies a regulatory role. One study showed a stimulatory role of these sequences on polyadenylation efficiency of α1(I) and α2(I) collagen mRNAs *in vitro*. This stimulation could be blocked by addition of RNA oligonucleotides containing the sequence of these elements, suggesting that there are *trans*-acting factors involved in regulation of collagen mRNA polyadenylation (Natalizio *et al.*, 2002). The identity of these factors and their role *in vivo* in determining preference of the polyadenylation sites is not known. The two α1(I) collagen mRNAs have different halflives (Maatta *et al.*, 1995) and may be translated with different efficacy, so preferential utilization of one polyadenylation site may regulate the amount of type I collagen synthesized.

4.1.1. *Regulation of α1(I) collagen mRNA stability*

The indication that α1(I) collagen mRNA may be regulated at the level of mRNA stability came when the halflife of α1(I) collagen mRNA was determined in fibroblasts to be longer than 12 hours (Hamalainen *et al.*, 1985; Ricupero *et al.*, 2001),

which is in the range of long-lived mRNAs. Treatment of cells with TGF-β, a known profibrotic cytokine, also increases the α1(I) collagen mRNA halflife (Penttinen *et al.*, 1988; Hellerbrand *et al.*, 1999; Kanzler *et al.*, 1999). Therefore, posttranscriptional regulation of α1(I) collagen gene expression was implicated. To estimate a relative contribution of transcriptional and posttranscriptional mechanisms on regulation in HSCs, the transcriptional rate of α1(I) collagen gene in HSCs was assessed by nuclear runoff assays (Stefanovic *et al.*, 1997). It was found that the transcription rate was increased only 3-fold in activated HSCs compared to quiescent HSCs, contrary to the 60–70-fold increase in the steady-state level of α1(I) collagen mRNA. The halflife of α1(I) collagen mRNA was subsequently determined and it was found that α1(I) collagen mRNA had a halflife of about 1.5 hours in quiescent HSCs and more than 24 hours in activated HSCs, an increase of about 16-fold (Stefanovic *et al.*, 1997). Together with increase in the transcription rate of the gene, such stabilization can account for the increase in steady-state level of α1(I) collagen mRNA (Stefanovic *et al.*, 1997). These experiments demonstrated that stabilization of the α1(I) collagen mRNA is an important mechanism, leading to dramatic accumulation of α1(I) collagen mRNA in activated HSCs. Regulation of α1(I) collagen mRNA stability in the HSC appears to be a complex process involving several regulatory elements (Fig. 1.8). The stability of most mRNAs is determined by *cis*-acting elements, which may be present in the 5' UTR, in the coding region, or in the 3' UTR (Ross, 1995; Guhaniyogi and Brewer, 2001). Regulation of the halflife of an mRNA is accomplished by sequence-specific interactions of protein factors with these elements in the mRNA (Bevilacqua *et al.*, 2003). Maata and coworkers see copy MS 35-36 synthesis (Stefanovic *et al.*, 2000).

The mechanism by which the 5' stem-loop targets mRNAs for turnover in HSCs and fibroblasts grown in three-dimensional matrix is not known. In quiescent HSCs protein binding was not detected to the 5' stem-loop *in vitro*; however, in activated HSCs a cytosolic protein factor(s) of 120 kD bound to the 5' stem-loop and required the 7 mG cap on the RNA for binding, consistent with this protein playing a role in promoting translation and/or stability of the mRNA (Stefanovic *et al.*, 1999). An excess of cap analogue completely prevents formation of this complex *in vitro*. The complex is also found in fibroblasts in postpolysomal cytoplasmic fraction. It is not known whether this complex interacts directly with the 7 mG cap or with the cap binding protein eIF4E (Hagedorn *et al.*, 1997). In quiescent HSCs we could detect no protein binding to the stem-loop *in vitro*. The binding of this complex is also greatly reduced if the cells are cultured in a three-dimensional matrix, when there is little collagen mRNA and collagen synthesis. Thus, if these cytoplasmic proteins are absent, as in quiescent HSCs or

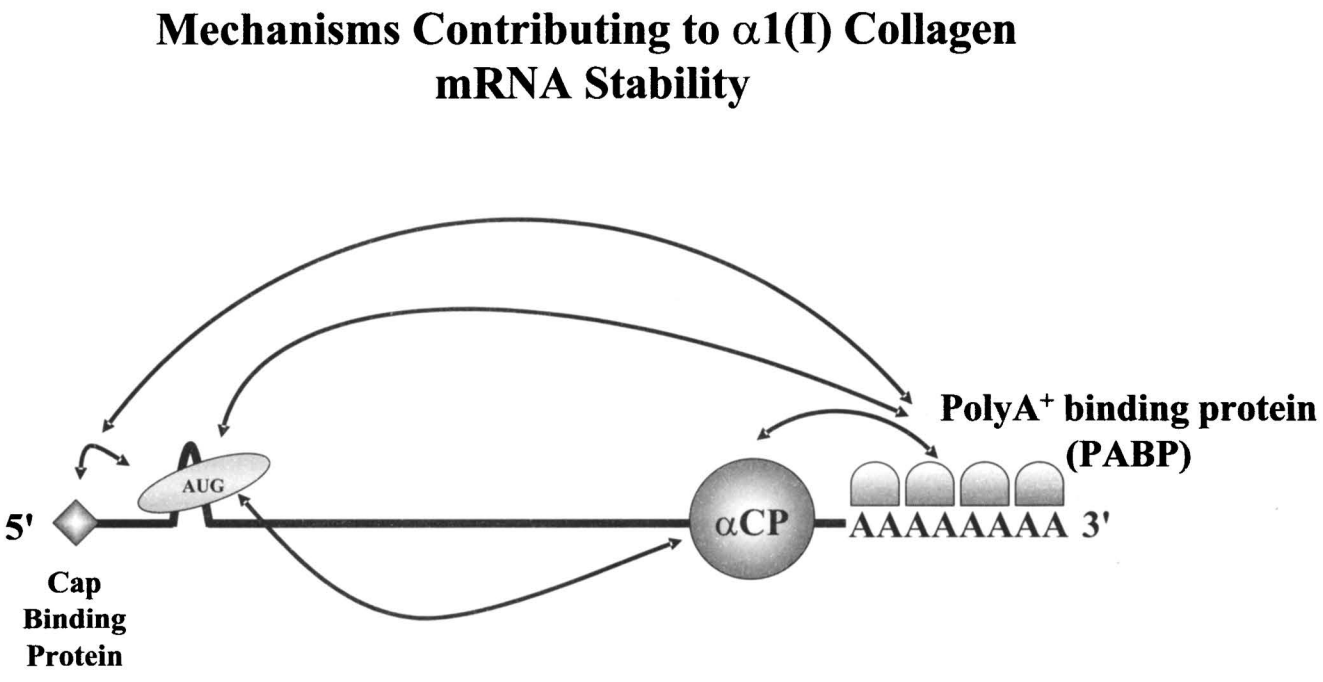

Fig. 1.8: Mechanisms influencing α1(I) collagen mRNA stability in the HSC. Multiple regulatory elements have been identified that contribute to stabilization of the α1(I) collagen mRNA in the HSC. These include the 5' Cap binding protein, a protein that interacts with the conserved 5' stem loop structure in the mature α1(I) collagen mRNA molecule, αCP binding to the 3' end of the mRNA molecule and poly A+ binding to the polyadenylation track at the end of the α1(I) collagen mRNA molecule. These all appear to interact in concert to influence message stabilization.

reduced, as in fibroblasts grown in gel, α1(I) collagen mRNA may be inefficiently translated and targeted for degradation. If they are present, as in activated HSCs and fibroblasts grown in plastic, the α1(I) collagen mRNA may be stabilized and directed for translation. Thus, the 5' stem-loop binding activity may increase the steady-state level of collagen mRNAs by diverting them from the degradative pathway.

Nuclear binding activity targeted to the α1(I) collagen stem-loop has also been reported (Stefanovic *et al.*, 2000). This activity does not require the presence of a 7 mG cap for binding and has a different electrophoretic mobility in native gels than the cytoplasmic binding activity. Nuclear binding correlated inversely with accumulation of α1(I) collagen mRNA, suggesting that it may be a negative modulator of α1(I) collagen mRNA expression acting at a nuclear step in mRNA metabolism. A protein of 30 kD has been shown to be cross-linked to the 5' stem-loop in nuclear extracts (our unpublished data). Clearly, a different protein binds the 5' stem-loop in the nucleus, suggesting that the 120 kD protein may replace it as the mRNA is exported into the cytoplasm. So, it is likely that α1(I) collagen mRNA is regulated by complex interactions with sequence-specific RNA binding proteins in both the nucleus and the cytoplasm. Translation and mRNA decay are coupled processes, so α1(I) collagen sequence-specific RNA binding proteins may be involved in regulation of both (Coller *et al.*, 1998). Therefore, studies on translation of α1(I) collagen mRNA are required to provide insight into the mechanism of stabilization of this mRNA.

4.2. *Regulation of α1(I) collagen mRNA translation*

One aspect for the regulation of protein synthesis that is often overlooked is the rate of translation. It may take up to 30 minutes to transcribe, process, and transport the average-sized mRNA, and several hours to accumulate large amounts of mRNA from a single gene. Translational regulation allows the cell to rapidly respond to changes in the environment by altering the rate of synthesis of specific proteins. In the normal cell, there is competition among the many mRNAs for the translation initiation factors, which clearly limits the translation process (Varenne *et al.*, 1984; Stefanovic and Brenner, 2003). Regulation of translation can then be accomplished by altering the efficiency of assembly of the initiation complex on the mRNA. An mRNA being efficiently translated has multiple ribosomes bound to it at any one time, with ribosomes located 80–100 nt apart (Singh, 1996). Translation occurs at about 5 amino acids s^{-1} (15 nt s^{-1}), meaning that the time between ribosomes is about 6 seconds. Every six seconds a ribosome completes the synthesis of a protein, so one mRNA molecule produces about 10 proteins^{-1} min (Scornik, 1974).

5' UTRs can regulate the rate of translation initiation and thus indirectly influence the stability of mRNA. In general, mRNAs with long and highly structured 5' UTRs are inefficiently translated. Stable stem-loops ($\Delta G > 50$ kcal^{-1} mol) or stem-loops that bind RNA binding proteins can block translation initiation if placed adjacent to the cap. This block is by steric hindrance, since RNA binding proteins normally not involved in translation showed this effect (Paraskeva *et al.*, 1998). Binding of iron regulatory proteins to the iron responsive element (IRE) in the 5' UTR of ferritin and erythroid 5-aminolevulinic acid synthase mRNA regulated their translation in response to the iron stores in the cell (Gray and Hentze, 1994). This regulation could be exerted only if the IRE was placed adjacent to the RNA cap. In the 5' UTRs of three collagen mRNAs, α1(I), α2(I) and α1(III), a stem-loop structure encompassing the translation initiation codon was found (Stefanovic *et al.*, 1999). This structure was located about 75 nt from the cap and had a stability of $\Delta G = 25$–30 kcal^{-1} mol in different collagen mRNAs. These features make it unlikely that it can regulate translation as described for the IRE.

High evolutionary conservation of the 5' stem-loop in vertebrate fibrillar collagen mRNAs strongly suggests a regulatory role. Since the start codon is part of this stem-loop, the sequence constraints required to maintain the 5' stem-loop dictate the sequence around translation initiation. Therefore, the start codon in collagen mRNAs is not in the sequence context necessary for optimal translation initiation rather it is surrounded by the sequence under evolutionary pressure to maintain the 5' stem-loop (Kozak, 1986; Kozak, 1997). The 5' stem-loop structure has a stability of 25–30 kcal mol^{-1}. This is insufficient to block scanning ribosomes to reach the start codon. Nevertheless, reporter mRNA with the collagen 5' stem-loop was translated *in vitro* 3-fold less efficiently than similar mRNA in which the 5' stem-loop was mutated (Stefanovic and Brenner, 2003). α1(I) collagen translation initiation site was even less efficient if another mRNA was added into the system to compete for the translation machinery. Reporter mRNA with the 5' stem-loop produced 25-fold less protein in the presence of a competitor mRNA than the reporter mRNA without the 5' stem-loop. When the sequence surrounding the start codon of α1(I) collagen mRNA was optimized to conform to Kozak rules and inserted in the reporter mRNA, translation increased 4-fold under competitive conditions (Stefanovic and Brenner, 2003). Thus, it seems that α1(I) collagen mRNA is designed to be inefficiently translated; the structure of the 5' stem-loop together with its suboptimal translation start site are both responsible for this effect. Being a poor translational substrate, this mRNA may be subjected to regulation by the 5' stem-loop RNA binding proteins.

A poorly understood mechanism of translational regulation utilizes small open reading frames located within the 5' UTR that control the rate of translation initiation. The functional significance of this mechanism has been documented in yeast, but only a few examples of this mechanism in mammals are available (Grant *et al.*, 1995; Mittag *et al.*, 1997; Child *et al.*, 1999; Eiznhamer *et al.*, 2001; Law *et al.*, 2001; Lee *et al.*, 2002; Raney *et al.*, 2002). About 10% of mammalian mRNAs, many of which are involved in regulation of cell growth, contain short open reading frames

in the 5' UTR (Kozak, 1987). These uORFs (upstream open reading frames) are often translated and regulation of the termination of their translation affects the translation of the downstream open reading frame. In extreme cases translation of the uORF in the mRNA was arrested with the mRNA associated with a single ribosome. Only when the ribosome had completed translation of the uORF was initiation of the downstream AUG activated. Regulation of the termination and release of the ribosome from the uORF accounted for the regulation of translation seen on these mRNAs (Grant *et al.*, 1995). Each of the three collagen mRNAs that have the 5' stem-loop (α1(I), α2(I), and α1(III)), also has two uORF. Thus it seems that the two features—5' stem-loop and uORFs—may act in concert. However, no changes in the translation of reporter mRNAs were observed *in vitro* when these uORF were abolished (Stefanovic and Brenner, 2003). Experiments *in vivo* will be necessary to confirm whether uORFs in fibrillar collagen mRNAs have a regulatory role on translation.

Upon secretion into extracellular space procollagen is cleaved by C-terminal endopeptidase (BMP-1) and N-terminal endopeptidase (ADAMTS) to yield collagen triple helical fibrillar core and two globular terminal peptides (Kivirikko, 1998). Some reports have suggested that N-terminal and C-terminal peptides of type I collagen inhibit translation of α1(I) collagen mRNA by reentering the cell and interacting with the translation apparatus (Paglia *et al.*, 1979; Wu *et al.*, 1986). However, we saw no inhibition when these recombinant peptides were added to *in-vitro* translation reactions or to cultured cells.

Type I procollagen is heterotrimeric secreted protein composed of two pro-α1 and one pro-α2 collagen polypeptides. Subcellular targeting of α1(I) and α2(I) collagen mRNAs and their coordinate translation have not been studied in detail. Collagens are secreted proteins and their translation is coupled to the export of the peptides into the endoplasmic reticulum (ER) (Kivirikko, 1998). It is not known whether association of the collagen translation apparatus with ER is due to targeting of the mRNA or targeting by the leader peptide after initiation of translation. All α1(I) collagen mRNA is associated with membrane bound polysomes and is not found on free polysomes or in postpolysomal supernatant (our result). Based on electron-microscopic data, assembly of collagen type I heterotrimer occurs on the membrane of endoplasmic reticulum, while the individual chains are still associated with polysomes or shortly after their release (Beck *et al.*, 1996; Veis and Brownell, 1977). Lysyl hydroxylase, one of the key enzymes responsible for posttranslational modification of type I collagen, is also associated with the membrane of endoplasmic reticulum (Suokas *et al.*, 2000). HSP 47 is a chaperone for folding of collagen triple helix (Nagata, 1996). This protein coimmunoprecipitates with the polysome associated α1(I) collagen chains, so it also targeted to the site of collagen translation (Koide *et al.*, 1999). Membrane association may couple folding starting from the C-terminus of collagen chains, to concomitant modifications of the selected lysine residues. For collagen type I, this implies that α1(I) and α2(I) chains may be synthesized by ribosomes positioned in close proximity on the ER membrane. Such coordinated translation would greatly increase local concentration of the chains. The sequence elements that modulate loading of ribosomes on α1(I) collagen mRNA may be involved in targeting for such coordinated translation. Therefore, we mutated the 5' stem-loop and analyzed production of collagen triple helices *in vivo* from the hybrid mouse-human collagen genes (Stefanovic and Brenner, 2003). These genes were driven by 220 nt of murine α1(I) collagen promoter, followed by mouse 5' UTR with the 5' stem-loop (5'WT-MH-COLL) or with mutated 5' stem-loop (5'MUT-MH-COLL), ligated in frame with human α1(I) collagen cDNA. Mutation of the 5' stem-loop did not change the coding sequence of the genes; they both encoded for identical procollagen polypeptide. The genes were expressed in Mov13 fibroblasts, which did not transcribe the endogenous α1(I) collagen gene, but contained all the other components of the collagen biosynthetic machinery (Hartung *et al.*, 1986; Stacey *et al.*, 1987). The 5'WT-MH-COLL gene produced triple helical collagen in Mov13 fibroblasts, which was resistant to digestion by pepsin. The 5'MUT-MH-COLL gene produced collagen that was sensitive to digestion with pepsin, suggesting that it was not in triple helical conformation (Monson, 1983). This structurally aberrant collagen was produced although the 5'MUT-MH-COLL gene contained the identical coding region. The pepsin-sensitive collagen may represent individual α1(I) collagen chains that were not efficiently folded into triple helix and were secreted as monomers or, alternatively, the monomers were not properly modified and an unstable triple helix was secreted. The 5'MUT-MH-COLL chains had identical electrophoretic mobility to 5'WT-MH-COLL chains, excluding a major difference in posttranslational modification, although subtle differences may have remained undetected. This result indicates that the 5' stem-loop couples translational machinery to the rest of collagen biosynthetic pathway and, to our knowledge, this is the first example of an RNA element that affects protein folding (Stefanovic and Brenner, 2003). Therefore, in the absence of RNA binding proteins, the 5' stem-loop renders collagen mRNAs inefficient for translation and therefore susceptible to regulation, such as by TGFβ (Narayanan *et al.*, 1989; Perr *et al.* 1996). In collagen-producing cells, the 5' stem-loop has a novel function of directing the posttranslational modification of collagen to produce mature triple helices.

Collagen biosynthesis requires coordinate action of translational apparatus, modifying enzymes and molecular chaperones. In human disease OI certain mutations of the α1(I) collagen chain decrease the rate of assembly of collagen type I. Interestingly, one patient with OI type I was described who had a mutation in the 5' stem-loop in the absence of any other mutation of α1(I) collagen gene (Willing *et al.*, 1995; Dalgleish, 1998). Unassembled OI α1(I) collagen chains are hypermodified on proline and lysine residues and degraded (Tajima *et al.*, 1994; Pace *et al.*, 2001). This suggests that modification and assembly processes are in a kinetic equilibrium. Unassembled procollagen polypeptides undergo

intracellular degradation by a proteosome dependent mechanism (Pace *et al.*, 2001; Stefanovic *et al.*, *Mol Cell Biol*, in press). This implies that unassembled procollagen chains must be retrotranslocated into the cytoplasm. How procollagen chains are targeted for destruction and returned to the cytoplasm is not known. One possibility is that the polypeptides remain associated with ER membrane until proper modifications are made and until they initiate productive folding into a triple helix. In such a case, misfolded or hypermodified chains would be reloaded into a translocon and reverse translocated into the cytoplasm for degradation. Elucidation of this mechanism has profound implications for how the biosynthesis of type I procollagen is regulated and if it takes place entirely on the membrane of the ER. Folding of type III procollagen could be achieved when its C-terminal domain was replaced with a single transmembrane domain of hemagglutinin (Bulleid *et al.*, 1997). Thus, procollagen folding can take place on the membrane of the ER. If procollagen synthesis is membrane bound, this would greatly increase local concentration of the components involved. Elucidation of all components of the translocation/synthesis machinery for type I collagen is therefore an important future goal.

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CYP2E1—Biochemical and Toxicological Aspects and Role in Alcohol-induced Liver Injury

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SUMMARY

Ethanol-induced oxidative stress appears to play a major role in mechanisms by which ethanol causes liver injury. Many pathways have been suggested to contribute to the ability of ethanol to induce a state of oxidative stress. One central pathway appears to be the induction of the CYP2E1 form of cytochrome P450 enzymes by ethanol. CYP2E1 is of interest because of its ability to metabolize and activate many toxicological substrates, including ethanol, to more reactive toxic products. Levels of CYP2E1 are elevated under a variety of physiological and pathophysiological conditions, and after acute and chronic alcohol treatment. CYP2E1 is also an effective generator of reactive oxygen species such as the superoxide anion radical and hydrogen peroxide, and in the presence of iron catalysts, produces powerful oxidants such as the hydroxyl radical. This review article summarizes some of the biochemical and toxicological properties of CYP2E1 and briefly describes the use of HepG2 cell lines developed to constitutively express the human CYP2E1 in assessing the actions of CYP2E1. Regulation of CYP2E1 is quite complex and briefly reviewed. Future directions and therapeutic considerations, which may help in understanding the actions of CYP2E1 and its role in alcoholic liver injury are suggested.

Keywords: CYP2E1; alcohol liver disease; oxidative stress; lipid peroxidation; HepG2 cells

1. Introduction

1.1. *Cytochrome P450 and oxidative stress*

The cytochrome P450 enzymes are a superfamily of heme proteins that serve as terminal oxidases in the mixed function oxidase system for metabolizing various endogenous substrates such as steroids and fatty acids, and xenobiotics including drugs, toxins, and carcinogens (Guengerich, 1987). Many different enzymes belong to this P450 family; P450s are present in virtually all living organisms. A systematic nomenclature system was developed for the P450 family based on the sequence identity of the various P450 sequences (Nebert *et al.*, 1991; Nelson *et al.*, 1996). The enzymes are named CYP for cytochrome P450, followed by an Arabic number denoting the family (more than 40% identity on the amino acid sequence level), a letter designating the subfamily (more than 55% identity) and finally an Arabic numeral representing the individual gene in the subfamily. The P450s catalyze many different chemical reactions including monooxygenation (insertion of an atom of oxygen into the substrate),

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peroxidation, reduction, dealkylation, epoxidation, and dehalogenation (Porter and Coon, 1991; Rendic and DiCarlo, 1997; Guengerich, 2001). Many different compounds of diverse structure can be metabolized by P450 enzymes. A major function of P450-catalyzed reactions is to convert a compound into a more polar metabolite that can be easily excreted directly by the organism or conjugated by phase II enzymes into more polar excretable metabolites. With some compounds, e.g. carbon tetrachloride or acetaminophen, metabolism by P450 can give rise to toxic metabolites which damage cells. For P450s to function catalytically, flavoprotein reductases such as NADPH-cytochrome P450 reductase, adrenodoxin, adrenodoxin reductase, are necessary to transfer electrons from NADPH or NADH to reduce the heme from the ferric redox state to the ferrous state. The latter is necessary to bind molecular oxygen to form the oxygenated P450 complex that catalyzes the diverse chemical reactions mentioned above (Lewis and Pratt, 1998). Cytochrome b5 may also play an important role in electron transfer to certain P450s.

It is important to recognize that oxygen activation by P450, necessary for enzyme catalytic function, can also result in the production of reactive oxygen species (ROS). Small amounts of the superoxide anion radical (O_2^-) can be produced from decay of the oxygenated P450 complex, while hydrogen peroxide (H_2O_2) can form from either dimutation of O_2^- or from decay of the peroxy P450 complex (Kuthan and Ullrich, 1982; White, 1991; Loida and Sligar, 1993). ROS have been implicated in many of the major diseases that plague mankind, including the toxicity of O_2 itself, hyperbaric O_2, ischemia-reperfusion injury, cardiovascular diseases, atherosclerosis, carcinogenesis, diabetes, neurodegenerative diseases, including Parkinson's disease and Alzheimer's disease, toxicity of heavy metals, e.g. iron, asbestos injury, radiation injury, vitamin deficiency, drug (e.g. redox cycling agents) toxicity, aging, inflammation, smoke toxicity, emphysema, and toxicity of acute and chronic ethanol treatment (Bondy, 1992; Nordmann *et al.*, 1992; Kehrer, 1993; Knight, 1998; Cederbaum, 2001). ROS can be produced from many systems in cells including the mitochondrial respiratory chain (Chance *et al.*, 1979); the cytochrome P450s (Blanck *et al.*, 1991; White, 1991); oxidative enzymes such as xanthine oxidase, aldehyde oxidase, cyclooxygenase, monoamine oxidase, the NADPH oxidase complex (DeGroot, 1994; Toykuni, 1999); autooxidation of heme proteins such as ferrohemoglobin or myoglobin or biochemicals such as catecholamines, quinones or tetrahydrobiopterins. In addition to these cellular sources of ROS, environmental sources of ROS include radiation, UV light, smoke, and certain drugs metabolized to radical intermediates or which can redox cycle. ROS are toxic to cells because they can react with most cellular macromolecules inactivating enzymes or denaturing proteins, causing DNA damage such as strand breaks, base removal or base modifications which can result in mutation, peroxidation of lipids which can result in destruction of biological membranes and produce reactive aldehydic products such as malondialdehyde or 4-hydroxynonenal (Nakazawa *et al.*, 1996, McCord, 1998). A variety of enzymatic and nonenzymatic mechanisms have evolved to protect cells against ROS, including the superoxide dismutases, which remove O_2^-, catalase and the glutathione (GSH) peroxidase system which remove H_2O_2; glutathione transferases which can remove reactive intermediates and lipid aldehydes, metallothioneins, heme oxygenase, thioredoxin which remove various ROS; ceruloplasmin and ferritin which help remove metals such as iron that promote oxidative reactions; nonenzymatic, low molecular weight antioxidants such as GSH itself, vitamin E, ascorbate (vitamin C), vitamin A, ubiquinone, uric acid, bilirubin (Yu, 1994; Halliwell, 1999). Oxidative stress or toxicity by ROS reflects a balance between the rates of production of ROS compared to the rates of removal of ROS plus repair of damaged cellular macromolecules. While excess ROS can cause toxicity, macrophages and neutrophils contain an NADPH oxidase which produces ROS to destroy foreign organisms (Rosen *et al.*, 1995), and the enzyme myeloperoxidase catalyzes a reaction between H_2O_2 and chloride to produce the powerful oxidant hypochlorite (bleach) to help destroy foreign invaders. In addition, ROS at low concentrations, especially H_2O_2, may be important in signal transduction mechanisms in cells, and thus be involved in cellular physiology and metabolism (Lander, 1997).

1.2. *Alcohol, oxidative stress, and cell injury*

The ability of acute and chronic ethanol treatment to increase production of reactive oxygen species and enhance peroxidation of lipids, protein, and DNA has been demonstrated in a variety of systems, cells, and species, including humans. Much has been learned about alcohol metabolism, the various enzymes and pathways involved, and how alcohol, directly via its metabolism, or indirectly via its solvent-like action affecting cellular membranes impacts on cell function. Yet, despite this tremendous growth in understanding alcohol metabolism and actions, the mechanism(s) by which alcohol causes cell injury are still not clear. A variety of leading mechanisms have been briefly summarized (Bondy, 1992; Nordmann *et. al.*, 1992; Cederbaum, 2001), and it is likely that many of them ultimately converge as they reflect a spectrum of the organism's response to the myriad of direct and indirect actions of alcohol. A major mechanism that is a focus of considerable research is the role of lipid peroxidation and oxidative stress in alcohol toxicity. Many pathways have been suggested to play a key role in how ethanol induces "oxidative stress". Some of these include redox state changes (decrease in the NAD^+/NADH redox ratio) produced as a result of ethanol oxidation by alcohol and aldehyde dehydrogenases; production of the reactive product acetaldehyde as a consequence of ethanol oxidation by all major oxidative pathways; damage to mitochondria which results in decreased ATP production; direct or membrane effects caused by hydrophobic ethanol interaction with either phospholipids or protein components or enzymes; ethanol-induced hypoxia, especially in the pericentral zone of the liver acinus as oxygen is consumed in order for the liver to detoxify ethanol via oxidation; ethanol effects on the immune system,

and altered cytokine production; ethanol-induced increase in bacterial-derived endotoxin with subsequent activation of Kupffer cells; ethanol induction of CYP2E1; ethanol mobilization of iron which results in enhanced levels of low molecular weight nonheme iron; effects on antioxidant enzymes and chemicals, in particular mitochondrial and cytosolic glutathione; one electron oxidation of ethanol to the 1-hydroxy ethyl radical; and conversion of xanthine dehydrogenase to the xanthine oxidase form. Again, many of these pathways are not exclusive of one another and it is likely that several, indeed many systems contribute to the ability of ethanol to induce a state of oxidative stress.

What is the evidence that ethanol-induced oxidative stress plays a role in cell injury? While many studies have shown increases in lipid peroxidation or protein carbonyl formation by alcohol, it is not always clear whether these are causes of or consequences of the alcohol-induced tissue injury. Nevertheless, there are many studies which show that administration of antioxidants or iron chelators or GSH-replenishing agents can prevent or ameliorate the toxic actions of alcohol. The most convincing data that oxidative stress contributes to alcohol-induced liver injury comes from the studies using the intragastric infusion model of alcohol administration. In these studies, alcohol-induced liver injury was associated with enhanced lipid peroxidation, protein carbonyl formation, formation of the 1-hydroxyethyl radical, formation of lipid radicals, decreases in hepatic antioxidant defense especially GSH, (Morimoto *et al.*, 1994; Nanji *et al.*, 1994; Knecht *et al.*, 1995; Iimuro *et al.*, 2000; Tsukamoto and Lu, 2001). Replacement of polyunsaturated fat (required for lipid peroxidation to occur) with saturated fat or medium chain triglycerides in the diets fed to rats intragastrically, lowered or prevented lipid peroxidation, and alcohol-induced liver injury. Thus, alcohol plus polyunsaturated fat was required for injury to occur. Addition of iron, known to generate $^{\bullet}OH$ and promote oxidative stress, to these diets exacerbated liver injury (Tsukamoto *et al.*, 1995). Importantly, addition of antioxidants such as vitamin E, ebselen, superoxide dismutase, and GSH precursors, prevented alcohol-induced liver injury.

In addition to these *in-vivo* studies, *in-vitro* studies with hepatocytes also showed that ethanol can produce oxidative stress and hepatocyte toxicity. Studies with isolated hepatocytes from control rats or chronic ethanol-fed rats indicated that ethanol metabolism via alcohol dehydrogenase results in an increase in ROS production, hepatocyte injury, and apoptosis, reactions blocked by antioxidants (Adachi and Ishii, 2002; Bailey and Cunningham, 2002). Studies in our laboratory with HepG2 cell lines expressing CYP2E1 showed that addition of ethanol or polyunsaturated fatty acids or iron, or depletion of GSH, resulted in cell toxicity, increased oxidative stress, and mitochondrial damage, reactions prevented by antioxidants (Wu and Cederbaum, 1999). Since CYP2E1 plays a role in ethanol-induced oxidant stress and is a minor pathway of ethanol oxidation, the biochemical and toxicological properties of CYP2E1 constitute the basis for the remainder of this review.

2. CYP2E1

2.1. *CYP2E1 and the microsomal ethanol oxidizing system*

Alcohol dehydrogenase is the major enzyme pathway for oxidizing ethanol to acetaldehyde. Morphological observations that chronic ethanol treatment caused proliferation of the liver smooth endoplasmic reticulum suggested that ethanol, similar to certain xenobiotics which are metabolized by cytochrome P450, may also be metabolized by P450 (Lieber, 1999). A microsomal ethanol oxidizing system (MEOS) was characterized by Lieber and associates and shown to be dependent on P450 (Lieber, 1997). The Km for ethanol oxidation by the MEOS (about 10 mM) was about an order of magnitude greater than the Km for ethanol by alcohol dehydrogenase. Acetaldehyde is the product resulting from ethanol oxidation by MEOS and it is clear that MEOS represents a minor pathway of ethanol oxidation, probably accounting for less than 10% of the liver capacity to oxidize ethanol (Lieber and DeCarli, 1972). Importantly, activity of MEOS is enhanced after chronic ethanol treatment, partly due to an increased total content of P450, and partly due to induction of CYP2E1, a member of the P450 family with high catalytic activity with ethanol (Lieber, 1997). Induction of MEOS may play an important role in the metabolic tolerance found after chronic ethanol treatment, i.e., the increased capacity to oxidize ethanol. While there was controversy earlier over the nature of MEOS, purification of an ethanol-inducible form of P450 from rabbit liver microsomes firmly established the role of P450 in MEOS (Koop *et al.*, 1982). Ethanol-inducible P450s have been isolated from many species and while several P450s may be induced by ethanol, the major inducible P450 is now referred to as CYP2E1.

2.2. *CYP2E1 localization*

CYP2E1 is mainly found in the liver but significant amounts are also found in most organs, including the brain (Hansson *et al.*, 1990). Within the liver acinus, levels of CYP2E1 are highest in the centrilobular zone of the liver (Ingelman-Sundberg *et al.*, 1988). This is of interest because toxins such as ethanol, acetaminophen, nitrosamines, and carbon tetrachloride preferentially destroy the centrilobular liver region. CYP2E1 is expressed mainly in the hepatocytes of the liver; however, significant amounts are also found in the Kupffer cells (Koop *et al.*, 1991), and hepatocyte and Kupffer cell CYP2E1 are inducible e.g. by ethanol. CYP2E1, like other xenobiotic metabolizing P450s, is mainly located in the membrane of the endoplasmic reticulum (ER), where it is anchored and retained through its hydrophobic NH_2-terminus, leaving the large COOH-terminal domain including the catalytic site exposed to the cytosol. CYP2E1 has also been detected in other cellular compartments such as the plasma membrane (Loepper *et al.*, 1990, 1993; Wu and Cederbaum, 1992). CYP2E1 located at the plasma membrane has been suggested to play a role in the immune mediated

hepatotoxicity observed in patients suffering from ALD (Bourdi *et al.*, 1996; Eliasson and Kenna, 1996; Lytton *et al.*, 1999). CYP2E1 was shown to be transported out of the ER to the Golgi apparatus, with subsequent transfer to the plasma membrane (Neve *et al.*, 1996; Neve and Ingelman-Sundberg, 2000). Removal or modification of the hydrophobic NH_2-terminal transmembrane domain of CYP2E1 resulted in specific targeting to the mitochondria. After mitochondrial import and processing, a soluble and catalytically active protein, called Δ2E1 (Mr = 40 κD), was formed. Low levels of Δ2E1 were also observed in mitochondria isolated from rat liver, thus showing that Δ2E1 is present *in vivo*. Removal or modification of the NH_2-terminus of CYP2E1 resulted in exposure of a mitochondrial targeting signal that directs the protein to the mitochondria. The mitochondrial targeting signal was identified and demonstrated to be located between amino acid residues 74 and 95, an area rich in positively charged amino acid residues and also containing a hydrophobic region (Neve and Ingelman-Sundberg, 1999).

2.3. *CYP2E1 substrates*

Perhaps the most important endogenous substrate of CYP2E1 is acetone which is converted to acetol and methylglyoxal, three carbon intermediates which can ultimately produce glucose (Koop and Casazza, 1985). Blood levels of acetone under fasting conditions were elevated up to 50-fold in transgenic mice lacking CYP2E1 compared to wild type mice, indicating the critical role of CYP2E1 in acetone metabolism (Bondoc *et al.*, 1999). Other endogenous compounds that can be metabolized by CYP2E1 include fatty acids such as linoleic and arachidonic acids (Laethem *et al.*, 1993) and lipid peroxidation derived hydrocarbon gases such as pentane and hexane (Terelius and Ingelman-Sundberg, 1986). With respect to exogenous substrates, CYP2E1 metabolizes a variety of small, hydrophobic substrates including solvents such as chloroform and carbon tetrachloride, aromatic hydrocarbons such as benzene and toluene, alcohols such as ethanol and pentanol, aldehydes such as acetaldehyde, halogenated anesthetics such as enflurane and halothane, nitrosamines such as N,N-dimethylnitrosamine and drugs such as chlorzoxazone and acetaminophen. Table 2.1, adapted from Koop (1992), Raucy *et al.* (1993), Ronis *et al.* (1996), Lieber (1997), Tanaka *et al.* (2000), Bolt *et al.* (2003), summarizes some of the substrates which are effectively metabolized by CYP2E1. From a toxicological point of view, interest in CYP2E1 revolves around the ability of this P450 to metabolize and activate many toxicologically important compounds such as ethanol, carbon tetrachloride, acetaminophen, benzene, halothane and many other halogenated substrates. Procarcinogens including nitrosamines and azo compounds are effective substrates for CYP2E1, e.g. CYP2E1 is a low Km dimethylnitrosamine demethylase in contrast to other forms of P450 which exhibit a high Km for dimethylnitrosamine (Yang *et al.*, 1990). Toxicity by the aforesaid compounds is enhanced after induction of CYP2E1, e.g. by ethanol treatment, and toxicity is reduced by inhibitors of CYP2E1 or in CYP2E1 knockout mice (Lee *et al.*, 1996). Of the substrates mentioned in Table 2.1, chlorzoxazone is of special value as its hydroxylated product can readily be assayed in the blood and the ratio of 6-hydroxychlorzoxazone/chlorzoxazone is widely used to assess the approximate levels of CYP2E1 in humans, including alcoholics (Girre *et al.*, 1994).

Molecular oxygen itself is likely to be a most important substrate for CYP2E1. CYP2E1, relative to several other P450 enzymes, displays high NADPH oxidase activity as it appears to be poorly coupled with NADPH-cytochrome P450 reductase (Gorsky *et al.*, 1984; Ekstrom and Ingelman-Sundberg, 1989). CYP2E1 was the most efficient P450 enzyme in the initiation of NADPH-dependent lipid peroxidation in reconstituted membranes among five different P450 forms investigated. Furthermore, anti-CYP2E1 IgG inhibited microsomal oxidase activity and microsomal lipid peroxidation dependent on P450, but not lipid peroxidation initiated by the action of NADPH-cytochrome P450 reductase (Ekstrom and Ingelman-Sundberg, 1989). In our laboratory, we found that microsomes isolated from rats fed ethanol chronically were about twofold to threefold more reactive in generating superoxide radical and H_2O_2, and in the presence of ferric complexes, in generating hydroxyl radical and undergoing lipid peroxidation (Klein *et al.*, 1983; Dicker and Cederbaum, 1987; Puntarulo and Cederbaum, 1988; Rashba-Step *et al.*, 1993). CYP2E1 levels were elevated about threefold to fivefold in the liver microsomes after feeding rats the Lieber-DeCarli diet for four weeks. Mixed-function oxidase systems produce an oxidant that reacts with proteins to inactivate them and to generate protein carbonyls, a consequence of oxidation of certain amino acids such as histidine residues. This inactivation sensitizes the protein for eventual degradation by cellular proteinases and is important in protein turnover. Microsomes isolated from ethanol-fed rats were twice as reactive as those isolated from pair-fed or chow-fed rats in catalyzing inactivation of added enzymes, such as lactic dehydrogenase, alcohol dehydrogenase, or pyruvic kinase, in the presence of ferric-ATP or ferric-citrate (Dicker and Cederbaum, 1988). Similarly, microsomes isolated from ethanol-fed rats were more reactive in catalyzing DNA strand cleavage when plasmid DNA was added to the microsomal incubation system (Kukielka and Cederbaum, 1994).

In all the above systems, enhanced effectiveness of microsomes isolated from ethanol-fed rats was prevented by addition of chemical inhibitors of CYP2E1 and by polyclonal antibody raised against CYP2E1 purified from pyrazole-treated rats, confirming that the increased activity in these microsomes was due to CYP2E1. Validation for the production of the appropriate oxidant (e.g., hydroxyl radical) was obtained by use of appropriate antioxidant molecules (e.g., catalase, superoxide dismutase, competitive hydroxyl radical-scavenging agents, vitamin E, glutathione).

CYP2E1 is a minor pathway of ethanol oxidation as it catalyzes the two electron oxidation of ethanol to acetaldehyde. Interestingly, acetaldehyde is also a substrate for CYP2E1 and is oxidized to acetate; thus CYP2E1 can, at least theoretically, catalyze the oxidation of ethanol to acetate (Terelius *et al.*, 1991). However, this oxidation is likely to be

Table 2.1. Substrates metabolized/activated by CYP2E1

Alcohols, aldehydes, ketones	Aromatic compounds	Ethers	Fatty acids	Halogenated and nonhalogenated alkanes and alkenes	Nitrosamines, azocompounds	Reducible substrates
Acetaldehyde	Acetaminophen	Diethyl ether	Arachidonic acid ω-1	Acetoacetate	Azoxymethane	*t*-Butylhydroxyperoxide
Butanol	Aniline	Methyl *t*-butyl ether	and ω-2 hydroxylation	Acetol	N,N-Diethylnitrosamine	Carbon tetrachloride
2-Butanone	Benzene		Lauric acid	Acetone	N,N-Dimethylnitrosamine	Chromium [Cr(VI)]
Ethanol	Bromobenzene		ω-1 hydroxylation	Acetonitrile (+ catalase)	Methylazoxymethanol	Oxygen
Glycerol	Caffeine			Acrylonitrile	N-Nitroso-2,3-dimethylmorpholine	
Isopropanol	Capsaicin			1,3-Butadiene	N-Nitrosopyrrolidine	
Methanol	Chlorzoxazone			Chloroform		
Propanol	3-Hydroxypyridine			Chloromethane		
Pentanol	Isoniazid			Dibromoethane		
	Phenol			Dichloromethane		
	Pyridine			1,1-Dichloroethane		
	p-Nitrophenol			1,1-Dichloroethylene		
	Pyrazole			1,2-Dichloropropane		
	Styrene			N,N-Dimethylacetamide		
	Tamoxifen			N,N-Dimethylformamide		
	Toluene			Enflurane		
				Ethane		
				Ethyl carbamate		
				Ethylene dichloride		
				Halothane		
				Hexane		
				Methylenechloride		
				N-Methylformamide		
				Pentane		
				Thioacetamide		
				Trichloroethylene		
				Vinyl chloride		
				Vinyl bromide		

negligible in the presence of ethanol, the substrate which generates acetaldehyde (Wu *et al.*, 1998). CYP2E1 can also promote the one electron oxidation of ethanol to the 1-hydroxyethyl radical (Albano *et al.*, 1987, 1991). The mechanism for this oxidation is not clear but appears to involve the oxidase activity of CYP2E1 rather than a direct metabolism by CYP2E1, since production of the hydroxyethyl radical is inhibited by ROS scavengers such as superoxide dismutase and catalase (Knecht *et al.*, 1993 Rao *et al.*, 1996). Detection of the 1-hydroxyethyl radical in the bile after administration of ethanol to rodents has been a most valuable assay for determining ethanol-induced radical formation and oxidant stress *in vivo* (Reinke *et al.*, 1987; Knecht *et al.*, 1995). Formation of the 1-hydroxyethyl radical may play a role in the development of liver damage produced by ethanol, perhaps via formation of protein adducts with subsequent autoantibody formation (Albano *et al.*, 1996; Clot *et al.*, 1996).

2.4. *CYP2E1 polymorphisms*

The CYP2E1 gene is polymorphically distributed and 10 polymorphic loci on the human CYP2E1 gene have been reported (Harada *et al.*, 2001). No significant difference was observed in drinking behavior and susceptibility to alcohol in terms of frequencies of genotypes and allele among Japanese subjects (Itoga *et al.*, 1999). To date, there appears to be no relationship between allelic forms of the CYP2E1 gene and the incidence of ethanol-mediated liver cirrhosis. No differences in gene expression were observed when reporter constructs of various CYP2E1 variant alleles were transfected into transformed cell lines (Badger *et al.*, 2003). Many epidemiologic studies have been carried out to try to relate various CYP2E1 polymorphic alleles to numerous types of cancers (stomach, lung, colorectal, pancreatic, hepatic, renal, oval), but obvious and significant relationships have been difficult to observe. For a recent review on CYP2E1 polymorphisms, the reader is referred to Bolt *et al.* (2003).

2.5. *Induction of CYP2E1*

Many of the substrates for CYP2E1 can induce their own metabolism. This was initially observed with ethanol, a substrate for CYP2E1 that elevates CYP2E1 levels (Lieber, 1997, 1999). In fact, these two properties explain the ability of ethanol to inhibit metabolism of certain substrates when alcohol is present, i.e. ethanol and the substrate compete for oxidation by CYP2E1, and for ethanol to increase the metabolism of substrates when it is no longer present to compete, but the ethanol treatment elevated the levels of the CYP2E1 catalyst. Ethanol can be oxidized by other P450s besides CYP2E1, notably CYPs 3A and IA, and ethanol treatment can elevate the levels of these CYPs (Asai *et al.*, 1996; Salmela *et al.*, 1998). A variety of heterocyclic compounds such as imidazole, pyridine, pyrazole, 4-methylpyrazole, thiazole, isoniazid have been shown to elevate CYP2E1 levels as do solvents such as dimethylsulfoxide, various alcohols, benzene and acetone (Song *et al.*, 1986; Song, 1995; Song *et al.*, 1996). These low molecular weight compounds have been used *in vivo* or *in vitro* to elevate or help prevent loss of CYP2E1 under tissue culture conditions and their mode of mechanism is discussed below.

CYP2E1 can also be induced under a variety of metabolic or nutritional conditions. For example, CYP2E1 levels were elevated in chronically obese, overfed rats and in rats fed a high fat diet (Raucy *et al.*, 1991; Yun *et al.*, 1992). Somewhat paradoxical, in rats levels of CYP2E1 were increased by fasting and prolonged starvation (Hong *et al.*, 1987; Johansson *et al.*, 1990). Diabetes has been reported to increase expression of CYP2E1 mRNA and protein levels several fold (Woodcroft *et al.*, 2002). This may be related to actions of insulin which downregulated CYP2E1 expression at the posttranscriptional level in a rat hepatoma cell line (DeWaziers *et al.*, 1995; Peng and Coon, 1998) and in rat hepatocyte culture (Woodcroft and Novak, 1997). CYP2E1 levels were elevated in liver and kidney microsomes of rats treated with streptozocin (Shimojo *et al.*, 1993). CYP2E1 induction in diabetes may be associated with the elevated production of ketone bodies (Bellward *et al.*, 1987). The carbohydrate content of the diet influences CYP2E1 levels as a low carbohydrate diet increased the extent of induction of MEOS by ethanol (Teschke *et al.*, 1981) and high fat/low carbohydrate diets resulted in the highest levels of CYP2E1 induced by ethanol (Yoo *et al.*, 1991). In this respect, it is interesting that alcohol-induced liver injury is magnified in diets with very low levels of carbohydrate and high levels of fat (Badger *et al.*, 1998; Lindros and Jarvelainen, 1998).

Besides insulin, other hormones can affect CYP2E1 levels. Hypophysectomy and triiodothyronine increase CYP2E1 protein and mRNA levels in contrast to insulin which lowers them (Hong *et al.*, 1990; Peng and Coon, 1998). In primary rat hepatocyte cultures, glucagon lowered CYP2E1 levels by accelerating turnover of the CYP2E1 protein by a cyclic AMP-dependent process (Eliasson *et al.*, 1992). Testosterone increased renal but not hepatic, CYP2E1 levels (Hoivik *et al.*, 1995).

Nonalcoholic steatohepatitis (NASH) is a condition characterized by hepatomegaly, elevated serum aminotransferase levels, and a histologic picture similar to alcoholic hepatitis (Reid, 2001). Oxidative stress and lipid peroxidation is one of the critical factors involved in the genesis and probably progression of NASH (Weltman *et al.*, 1998). In a mouse model of NASH, hepatic CYP2E1 was upregulated and this was associated with a dramatic increase in total lipid peroxide levels that were substantially inhibited by anti-CYP2E1 antibody (Leclerq *et al.*, 2000). However, it is now recognized that NASH also develops in CYP2E1 knockout mice as other CYPs, notably CYP4A10 and CYP4A14 were upregulated (Leclerq *et al.*, 2000). An integrated concept whereby either CYP2E1 or CYP4A or both play key roles in ROS production and contribute centrally to the pathogenesis of NASH was recently proposed (Robertson *et al.*, 2001).

2.6. *Regulation of CYP2E1*

Considerable data have been reported elucidating the molecular mechanism of CYP2E1 regulation by exogenous compounds as well as during pathophysiological conditions. CYP2E1 is regulated by multiple, distinct regulatory mechanisms (Koop and Tierney, 1990; Ronis *et al.*, 1991; Song, 1995; Song *et al.*, 1996). The CYP2E1 gene is under transcriptional control during development. In rats, it is activated immediately after birth and is maximally transcribed within the first week. Upon fasting or induced diabetes, the mRNA for 2E1 is increased severalfold (Song *et al.*, 1987). It was suggested, from nuclear run-on transcription assays, that this increase is due to posttranscriptional mRNA stabilization. Although these mechanisms have been established, regulation of CYP2E1 by the small diverse group of chemicals is less clear. At one fixed time point (24 h) after administration of ethanol, acetone or pyrazole to rats, Song *et al.* found that mRNA levels did not increase (Song *et al.*, 1986). The mechanism of induction was therefore suggested to be at the level of protein degradation. Unlike other major classes of cytochrome P450 (especially, class I through IV family members) which can be transcriptionally activated by their respective inducers, CYP2E1 is not transcriptionally activated by an acute bolus dose or chronic administration of ethanol, acetone, or other exogenous inducing agents. Although elevation of CYP2E1 mRNA levels has been reported (Tsutsumi *et al.*, 1989), most investigators reported little induction or slight reduction of CYP2E1 mRNA level after ethanol administration (Song *et al.*, 1986, 1996; Johansson *et al.*, 1988). From *in-vivo* data of CYP2E1 turnover in rats chronically treated with acetone (Song *et al.*, 1989) and *in-vitro* hepatocyte culture systems (Eliasson *et al.*, 1988; Wu *et al.*, 1990), exogenous CYP2E1 inducers such as acetone, ethanol, imidazole and 4-methylpyrazole were shown to increase CYP2E1 by protein stabilization. Using *in-vivo* radiolabeling of CYP2E1 followed by immuno-purification, the turnover rate of CYP2E1 was determined. Under normal conditions, CYP2E1 was degraded by two phases with a short halflife of 7 h and a longer halflife of 37 h. However, after chronic acetone administration, the rapid phase of degradation was abolished, while the slower phase with the same halflife of 37 h was still observed. These data indicated that acetone, a substrate of CYP2E1, stabilizes the enzyme by inhibiting the rapid phase of its degradation (Song *et al.*, 1989).

However, different results have been reported to suggest alternative mechanisms of CYP2E1 regulation. Tsutsumi *et al.* (1993) reported that ethanol induces CYP2E1 by increasing the rate of CYP2E1 protein synthesis without changing its halflife (a single halflife of 27 h). It was also suggested that CYP2E1 may be degraded in the endoplasmic reticulum (Eliasson *et al.*, 1990, 1992). Furthermore, ethanol, at high concentrations (>300 mg per dl) achieved by the intragastric infusion model, induced the CYP2E1 gene transcriptionally (Ronis *et al.*, 1993). It is of interest to note that ethanol (up to 1.5 M concentration) did not change the level of CYP2E1 mRNA despite a fivefold elevation of CYP2E1 protein and its activity in a FGC-4 hepatoma culture system (McGhee *et al.*, 1994). The various differences discussed above may result from different model systems utilized. Using an *in-vivo* rat model of alcohol withdrawal, Roberts *et al.* (1994, 1995a) recently reported that ethanol increases CYP2E1 by protein stabilization. This phenomenon was observed not only in the liver but also other extrahepatic tissues such as kidney, brain, and intestine. In addition, CYP2E1 protein stabilization appeared dependent upon blood alcohol or acetone concentration. Furthermore, a turnover study, using *in-vivo* radiolabeling of CYP2E1 with (^{14}C) $NAHCO_3$ and immuno-purification, demonstrated that ethanol treatment abolished the rapid phase of CYP2E1 degradation while biphasic degradation of CYP2E1 was observed in the control animals (Roberts *et al.*, 1995b).

Our laboratory has investigated the increase in CYP2E1 levels by ligands such as pyrazole or 4-methylpyrazole (4-MP). Under a variety of reaction conditions (dose, time after *in-vivo* administration) or coadministration with streptozotocin which stabilizes CYP2E1 mRNA, or administration to neonates in which active CYP2E1 transcription is occurring, pyrazole and 4-MP elevated liver and kidney CYP2E1 immunoreactive protein and catalytic activity in the absence of an increase in CYP2E1 mRNA levels (Winters and Cederbaum, 1992; Wu and Cederbaum, 1993a, 1993b). In isolated rat hepatocyte cultures, CYP2E1 mRNA and protein levels and CYP2E1 catalytic activity rapidly declined with time in culture. Addition of pyrazole or 4-MP slowed the decline in CYP2E1 protein and activity, with no effect on CYP2E1 mRNA levels (Wu *et al.*, 1990). Similarly, McGhee *et al.* (1994) reported the halflife of CYP2E1 in a hepatoma cell line to be 1.8 h in the absence of ethanol and 45 h in the presence of ethanol. In our HepG2 cell model, the halflife of CYP2E1 was about 3 h, which was increased by glycerol, DMSO, ethanol, and 4-MP (Yang and Cederbaum, 1997a). It is clear that a major level of regulation of CYP2E1 formation appears to be posttranscriptional as various substrates and ligands increase the content of CYP2E1 by protection against rapid degradation by intracellular proteolytic pathways.

What triggers CYP2E1 turnover and the nature of the proteases responsible for its degradation is not clear. Two different pathways have been reported in the literature. Ingelman-Sundberg and coworkers reported that cAMP-dependent phosphorylation of CYP2E1 is followed by heme loss and subsequent apoprotein degradation by serine proteases present in the ER which exhibit proteolytic activities *in vitro*, toward detergent solubilized rat liver CYP2E1 (Eliasson *et al.*, 1990, 1992; Zhukov *et al.*, 1993). Substrates and ligands are postulated to prevent CYP2E1 from rapid degradation by blocking the recognition sites of phosphorylation. Tierney *et al.* (1992) using an *in-vivo* mouse model showed that 2E1 inactivated by CCl_4 and 3-aminotriazole was rapidly removed from the endoplasmic reticulum. In this model they detected production of high-molecular-weight microsomal proteins after CCl_4 treatment believed to be ubiquitin conjugates. Roberts (1997) using an *in-vitro* microsomal system plus a 105,000 xg supernatant

fraction found that the loss of CYP2E1 was accompanied by the appearance of high-mol-wt material, some of which reacted with antiubiquitin IgG, suggesting that ubiquitin conjugates may target CYP2E1 for rapid proteolysis. CYP2E1 may be labilized by oxidant stress as inhibition of electron transfer by inhibition of the NADPH-cytochrome P450 reductase increased stability of CYP2E1 (Zhukov and Ingelman-Sundberg, 1999).

In our laboratory, CYP2E1 degradation was studied in HepG2 cells which stably and constitutively express CYP2E1. When the cells were incubated for two days in the presence of CYP2E1 ligands such as ethanol, DMSO, glycerol, pyrazole or 4–MP, CYP2E1 steady-state levels were increased severalfold as shown by immunoblot analysis and by enhanced oxidation of p-nitrophenol (Yang and Cederbaum, 1997a). Levels of cytochrome b_5 or activities of NADPH-cytochrome P450 reductase or NADH-cytochrome b_5 reductase were not affected by the ligand (glycerol) treatment, suggesting some selectivity for effects on CYP2E1 compared to other microsomal enzymes which comprise the mixed-function oxidase system (Yang and Cederbaum, 1997a, 1997b). Levels of CYP2E1 mRNA were not increased by the ligands. Expression of CYP2E1 mRNA is under control of the LTRs of the retroviral promoter in the HepG2 cells. These results showed that ethanol and other CYP2E1 ligands increase the content and activity of CYP2E1 in this HepG2 cell model with no effect on CYP2E1 mRNA. To evaluate CYP2E1 turnover in the HepG2 cells, pulse-chase experiments or addition of cycloheximide to inhibit new protein synthesis followed by examination of the degradation rates of the remaining CYP2E1 protein were carried out. The halflife of CYP2E1 was 3 (pulse-chase) or 5 (cycloheximide) hours in the absence of ligand, whereas in its presence 60 to 80% of the original PNP oxidation activity and CYP2E1 levels remained 24 h after addition of cycloheximide or after addition of excess cold methionine. In contrast to results with ethanol, DMSO, 4MP or glycerol, not all CYP2E1 substrates stabilize the enzyme against degradation; addition of 2 mM CCl_4 caused a marked increase in the loss of CYP2E1 catalytic activity and content (Yang and Cederbaum, 1997a). CCl_4 has long been known to inactivate CYP2E1 and enhance its degradation (Sohn *et al.*, 1991; Tierney *et al.*, 1992). These experiments appear to suggest that ethanol and the other ligands elevate CYP2E1 levels by stabilizing the enzyme against rapid proteolysis.

2.6.1. *CYP2E1 and the proteasome complex*

What are the proteolytic systems responsible for CYP2E1 turnover and prevented from their action on CYP2E1 by ethanol? Microsomes isolated from these cells showed a very slow rate of CYP2E1 degradation, suggesting that a protease in the ER is not a likely candidate for the rapid degradation, of CYP2E1. Several inhibitors of lysosomal protease had little or no effect on CYP2E1 degradation. In order to evaluate a role for the proteasome complex in the degradation of CYP2E1, the effect of the substrate analogue Czb-I1e-Glu (otBu)-Ala-Leucinal (PSI) on CYP2E1 degradation and content was determined. PSI inhibited the chymotrypsin-like activity of the proteasome. Calpeptin inhibitor, a peptidyl aldehydic nonproteasomal cytosolic protease inhibitor, was also evaluated to try to minimize the possibility of nonspecific effects by PSI. PSI proved to be effective in preventing CYP2E1 degradation, whereas calpeptin inhibitor provided little or no protection (Yang and Cederbaum, 1997a). PSI added *in vitro* at concentrations up to 300 μM, had no effect on oxidation of PNP by microsomes, suggesting that PSI was not acting as a ligand or substrate for CYP2E1. Theoretically, one consequence of diminishing the proteolytic degradation rate of CYP2E1 by PSI should be the steady-state accumulation of CYP2E1 within the cells. Treatment of the HepG2 cells with PSI caused a concentration-dependent increase in levels of CYP2E1 apoprotein. Taken as a whole, these results indicate that the human CYP2E1 has a short halflife span and substrates can significantly modify its turnover rate in intact cells. The proteasome proteolytic pathway appears to be involved in the degradation process of CYP2E1 in this model. Importantly, Bardag-Gorce *et al.* (2002) recently showed that the rapid loss of CYP2E1, which occurs *in vivo* after the ethanol inducer is withdrawn, could be blocked by the proteasome inhibitor PS-341, thus establishing the critical role of the proteasome in regulating CYP2E1 turnover *in vivo*.

In view of the importance of the proteasome complex in the turnover of CYP2E1, the effect of chronic ethanol treatment on activity or content of the proteasome would be important to determine, since inhibition of this degradation complex would cause an increase in CYP2E1 levels. Studies by French and collaborators indeed showed that chronic ethanol administration in the intragastric infusion model produced a decrease in activity of the proteasome (Donohue *et al.*, 1998; Fataccioli *et al.*, 1999). This inhibition may also cause oxidized, damaged proteins to accumulate in the liver and thereby contribute to ethanol-induced hepatomegaly. To specifically determine the role of CYP2E1 in the chronic ethanol-induced lowering of proteasome activity, CYP2E1 knockout mice were treated with ethanol; whereas ethanol lowered proteasomal activity in wild-type mice, activity was not affected in the CYP2E1 knockout mice (Bardag-Gorce et al., 2000). It was suggested that CYP2E1-dependent ROS production and lipid peroxidation may be responsible for the decrease in proteasome activity. We recently showed that in HepG2 cells which constitutively express CYP2E1, inhibition of the proteasome with lactacystin and other typical inhibitors potentiated the toxicity caused by addition of a polyunsaturated fatty acid, arachidonic acid, or by depletion of GSH (Perez and Cederbaum, 2003). Potentiation of toxicity by the proteasome inhibitors was associated with an increased oxidative damage as reflected by elevated lipid peroxidation, protein carbonyls and protein nitrotyrosine adducts and could be prevented by antioxidants. Lactacystin also potentiated arachidonic acid toxicity in hepatocytes isolated from pyrazole-treated rats with elevated levels of CYP2E1. Thus, proteasome plays an important role in CYP2E1 turnover and removal of oxidized, damaged proteins and subsequently in CYP2E1-mediated toxicity.

3. CYP2E1 and alcohol-induced liver injury

Since CYP2E1 can generate reactive oxygen species (ROS) during its catalytic cycle, and its levels are elevated by chronic treatment with ethanol, CYP2E1 has been suggested as a major contributor to ethanol-induced oxidant stress and to ethanol-induced liver injury. Initial suggestions for a role for CYP2E1 in alcoholic liver injury arose from studies with the intragastric model of ethanol feeding in which prominent induction of CYP2E1 occurs and in which significant alcohol liver injury occurred (Morimoto *et al.*, 1994; Nanji *et al.*, 1994; Tsukamoto *et al.*, 1995). In these models, large increases in microsomal lipid peroxidation have been observed and the ethanol-induced liver pathology has been shown to correlate with CYP2E1 levels and elevated lipid peroxidation (Castillo *et al.*, 1992; Ronis *et al.*, 1993; Morimoto *et al.*, 1994; Nanji *et al.*, 1994). Experimentally, a decrease in CYP2E1 induction was found to be associated with a reduction in alcohol-induced liver injury (French *et al.*, 1997; Kim *et al.*, 1997). CYP2E1 inhibitors such as diallyl sulfide (DAS) (Morimoto *et al.*, 1993), phenethyl isothiocyanate (PIC) (Morimoto *et al.*, 1995; Albano *et al.*, 1996) and chlormethiazole (Gouillon *et al.*, 2000), blocked the lipid peroxidation and ameliorated the pathologic changes in ethanol-fed rats. Polyenylphosphatidylcholine (PPC), another compound exerting anti-CYP2E1 properties (Aleynik *et al.*, 1999) was effective in opposing alcohol-induced oxidative stress (Lieber *et al.*, 1997). A strong association between dietary carbohydrate, enhanced CYP2E1 induction and hepatic necrosis was observed. No liver injury was found if carbohydrate levels were elevated (Korourian et al., 1999). It was concluded that diet is an important factor in toxicity mediated by ethanol because of modulation of the levels of CYP2E1 (Korourian et al., 1999). Ethanol consumption in liquid diets does not cause liver injury. However, micro and macrovesicular steatosis, occasional inflammatory foci and a threefold increase in transaminase levels was observed in a nutritionally adequate ethanol containing liquid diet with a carbohydrate content of 5.5%; no changes were found if the level of carbohydrate was elevated to 11% (Lindros and Jarvelainen, 1998; Li *et al.*, 2001). Thus, dietary and nutritional factors play a key role in the toxic actions of ethanol to the liver, in part, due to modulation of the levels of CYP2E1. Recently, a CYP2E1 transgenic mouse model was developed that overexpressed CYP2E1. When treated with ethanol, the CYP2E1 overexpressing mice displayed higher transaminase levels and histological features of liver injury compared with the control mice (Morgan *et al.*, 2002).

On the other hand, studies by Thurman and colleagues have presented powerful support for a role for endotoxin, activation of Kupffer cells and cytokines such as TNFá in the alcohol-induced liver injury found with the intragastric infusion model (Yin *et al.*, 1999; Wheeler and Thurman, 2001). They suggested that CYP2E1 may not play a role in alcohol liver injury based on studies with gadolinium chloride or CYP2E1 knockout mice (Koop *et al.*, 1997; Kono *et al.*, 1999). However, in contrast to their observations, others have reported that gadolinium chloride did indeed decrease levels of several P450 enzymes including CYP2E1, and lowered the induction of CYP2E1 by ethanol (Badger *et al.*, 1997; Jarvelainen *et al.*, 2000). With respect to the CYP2E1 knockouts, only "early" alcohol-induced injury was studied (Tsukamoto, 2000). Moreover, Leclercq *et al.* (2000) using the same knockout mice observed that other CYPs, notably CYP4A10 and CYP4A14, were upregulated in the CYP2E1 knockout but not the wild-type mice; these CYPs were, like CYP2E1, active generators of reactive oxygen and catalysts of lipid peroxidation, and in the absence of CYP2E1 served as alternative initiators of oxidative stress. Activity of NADPH-cytochrome P450 reductase was elevated 2.5-fold by alcohol treatment in the CYP2E1 knockouts. This enzyme can generate ROS; thus, as discussed by Tsukamoto (2000), induction by ethanol of other CYPs or of the reductase in the CYP2E1 knockout mice might have served as alternative sources of oxidative stress in these mice, especially in the absence of CYP2E1. Another possible consideration is that the relative level of CYP2E1 to total P450 in mice (12 pmol per mg protein for controls, 60 pmol per mg after ethanol treatment) is much lower than the CYP2E1 to total P450 ratio in rats (about 40 and 800 pmol per mg respectively) (Bardag-Gorce *et al.*, 2000; Badger *et al.*, 2003). However, it was recently suggested that the metabolism of several CYP substrates, including those for CYP2E1, was similar between mice and rats (Arteel, 2003). French and collaborators found that the ethanol-induced oxidative inactivation of the proteasome and increase in oxidized proteins (but not fatty liver) was completely prevented in these CYP2E1 knockout mice (Bardag-Gorce *et al.*, 2000). Clearly, further studies are necessary to resolve the aforementioned discrepancies. As noted earlier, it is likely that several mechanisms contribute to alcohol-induced liver injury, and that ethanol-induced oxidant stress is likely to arise form several sources, including CYP2E1, mitochondria and activated Kupffer cells.

4. Biochemical and toxicological properties of CYP2E1 in HEPG2 cells

As discussed above, major interest in CYP2E1 reflects the ability of this enzyme to oxidize ethanol; to generate reactive products from ethanol oxidation, e.g. acetaldehyde and the 1-hydroxyethyl radical; to activate various agents (CCl_4, acetaminophen, benzene, halothane, halogenated alkanes, alcohol) to reactive products; to generate reactive oxygen species; and to be "induced" by ethanol (as well as several low-mol-wt agents), and under a variety of nutritional and pathophysiological conditions. An approach our laboratory has utilized to try to understand basic effects and actions of CYP2E1 is to establish cell lines that constitutively express human CYP2E1. HepG2 cell lines, which overexpress CYP2E1, were established either by retroviral infection methods (MV2E1-9 cells or E9 cells) or by plasmid transfection methods (E47 cells) (Dai *et al.*, 1993; Chen and Cederbaum, 1998). Hepatotoxins such as $CC1_4$ or acetaminophen were more toxic in E9 cells than control HepG2 cells, validating the utility of

the model to study CYP2E1-dependent toxicity (Dai and Cederbaum, 1995a, 1995b). We have characterized the toxicity of ethanol, polyunsaturated fatty acids (PUFA) such as arachidonic acid (AA), and iron in the E9 and E47 cell lines (Wu and Cederbaum, 1996, 1999; Chen *et al.*, 1997; Sakurai and Cederbaum, 1998). Concentrations of ethanol or AA or iron which were toxic to the CYP2E1-expressing cells had no effect on control HepG2 cells not expressing CYP2E1 or HepG2 cells expressing a different P450, CYP3A4 (3A4 cells). Toxicity to CYP2E1-expressing cells was found when GSH was depleted by treatment with 1-buthionine sulfoximine (Wu and Cederbaum, 2001a). Inhibitors of CYP2E1 prevented toxicity by the aforesaid treatments. Antioxidants such as vitamin E, trolox and catalase also prevented toxicity. The aforementioned treatments of CYP2E1-expressing cells with ethanol, AA, iron or BSO resulted in an increase in oxidative stress to the cells as reflected by increased lipid peroxidation and enhanced dichlorofluorescein fluorescence. In other studies, we observed that (Caro and Cederbaum, 2001) low concentrations of iron and arachidonic acid that are not cytotoxic themselves can act as priming or sensitizing factors for CYP2E1-dependent loss of viability in HepG2 cells or rat hepatocytes. This synergistic toxicity was associated with elevated lipid peroxidation and could be prevented by antioxidants which preclude lipid peroxidation. Damage to mitochondria by CYP2E1-derived oxidants seems to be an early event in the overall pathway of cellular injury. Relatively low concentrations of iron or arachidonic acid were effective in promoting toxicity in the CYP2E1-expressing cells, supporting the suggestion that interactions between CYP2E1 and iron and polyunsaturated fatty acids may lower the threshold concentrations for these reactive nutrients for inducing a state of oxidative stress, which may play a role in the development of alcohol-induced liver injury.

To extend the results with the HepG2 cells to primary hepatocytes, cultures of hepatocytes from rats treated with pyrazole to elevate levels of CYP2E1 and from saline controls were evaluated. Many of the basic findings described above for E9 and E47 cells were recapitulated in the hepatocytes from the pyrazole-treated rats (called pyrazole hepatocytes), i.e. AA or iron or AA plus iron were more toxic to pyrazole hepatocytes than saline control hepatocytes (Wu and Cederbaum, 2000, 2001b; Caro and Cederbaum, 2001). Ethanol was weakly toxic, but even this was greater in the pyrazole hepatocytes. Toxicity was associated with increased lipid peroxidation and could be prevented by antioxidants and the CYP2E1 inhibitor diallylsulfide.

Adaptation to oxidant stimuli is critical for short- and long-term survival of cells exposed to oxidative stress. While much of the focus on CYP2E1 has been from a toxicology point of view, the possibility that the hepatocyte attempts to respond to increased levels of CYP2E1 by upregulation of protective factors has not been studied. We found, to our surprise, that the E47 cells had higher GSH levels than CYP3A4 expressing HepG2 cells or control HepG2 cells (Mari and Cederbaum, 2000). Increases in GSH were due to activation of the genes encoding the heavy and light subunits of gamma glutamyl cysteine synthetase (GCLC and GCLM). These increases in the E47 cells were prevented by inhibitors of CYP2E1 and were also found in hepatocytes from pyrazole-treated rats with elevated levels of CYP2E1 compared to saline-treated controls (Mari and Cederbaum, 2000; Nieto *et al.*, 2003). Increase in GSH and GCLC and GCLM mRNA was prevented by antioxidants, suggesting that ROS generated by CYP2E1 were responsible for the upregulation of these antioxidant genes. GCLC and GCLM mRNA expression and protein levels were further increased when E47 cells were challenged with substrates for CYP2E1 or prooxidants, which further elevated oxidative stress, such as ethanol, AA, and Fe+AA. The increase in mRNA in treated E47 cells was blocked by antioxidants and by a CYP2E1 inhibitor. The transcriptional upregulation mediated by CYP2E1-derived ROS seems to operate through a redox-sensitive element (ARE4) localized 3.1 kilobases upstream of the transcription start site in the GCLC gene (Nieto *et al.*, 2003). The mechanisms controlling the induction of genes by oxidative stress involve the activation of transcription factors, such as NF-kβ AP-1, and Nrf2, which could mediate such inductions (Morel and Barouki, 1999), but this remains to be evaluated.

There was also a two-fold increase in the content and activity of catalase, cytosolic glutathione transferase and microsomal glutathione transferase in the E47 cells due to activation of their respective genes (Mari and Cederbaum, 2001). These activations in the E47 cells were prevented by antioxidants, suggesting that ROS generated by CYP2E1 were responsible for the upregulation of these antioxidant genes. Indeed, the E47 cells, because of this activation of antioxidant genes, were less sensitive to toxicity by added H_2O_2 or menadione or 4-hydroxynonenal than were control cells. We believe that the upregulation of these antioxidant genes may reflect an adaptive mechanism to remove CYP2E1-derived oxidants.

Hepatic stellate cells (HSC) are central to the fibrotic response of the liver to injury, and ROS activate stellate cells. Since CYP2E1 produces ROS, ethanol-induced CYP2E1 expression may promote collagen type I biosynthesis by stellate cells. However, CYP2E1 is mostly present in hepatocytes, whereas stellate cells contain low levels of CYP2E1. Accordingly, a coculture model involving HepG2 cells and stellate cells was developed (Nieto et al., 2002a, 2002b) in which the HepG2 cells and the stellate cells were separated from each other by an insert; therefore, the experimental protocol was designed to evaluate whether mediators (ROS, cytokines, growth factors) produced by HepG2 cells diffuse to the HSC and affect collagen type I protein levels. There was a time-dependent increase in collagen type I when stellate cells were coincubated with C34 control cells, which was further elevated when stellate cells were coincubated with CYP2E1-overexpressing E47 cells. E47 plus stellate cell cocultures secreted much more type-I collagen protein. These experiments suggest that CYP2E1-overexpressing E47 cells generate diffusible mediators that promote type I collagen synthesis and release by stellate cells. Catalase and vitamin E markedly decreased type I collagen

synthesis by both cocultures and completely blocked the increased collagen production by the E47 coculture. These results suggest that E47 cells release ROS, such as H_2O_2 and lipid peroxidation products, that stimulate type-I collagen synthesis by stellate cells.

Work from several laboratories has indicated that mitochondrial damage may represent a common early event in cell injury caused by toxic agents (Susin *et al.*, 1998). Mitochondrial damage is initially manifested by a decrease in mitochondrial membrane potential ($\Delta\psi_m$) followed by ATP depletion (Orrenius *et al.*, 1996; Trost and Lemasters, 1996). Two major processes are likely candidates as the mechanism for a loss of $\Delta\psi_m$: nonspecific damage to the inner mitochondrial membrane or a more specific process, the permeability transition, due to the opening of mitochondrial permeability pore. Mitochondrial membrane potential was assessed by flow cytometry after double staining with rhodamine 123 and propidium iodide (PI). Exposure of E47 cells to BSO (Mari *et al.*, 2002), AA (Perez and Cederbaum, 2001), and Fe+AA (Caro and Cederbaum, 2001) increased the percentage of cells that showed low rhodamine 123 fluorescence but were not stained with PI. This population refers to cells that are still viable but with damaged mitochondria, showing that these CYP2E1-dependent models of toxicity affect mitochondria before the onset of cell death (i.e., early event). This early mitochondrial damage was prevented by antioxidants, linking oxidative stress to mitochondrial damage. That mitochondria are an important target for CYP2E1-mediated oxidative stress is suggested by the fact that overexpression of mitochondrial catalase is capable of protecting cells that overexpress CYP2E1 against the toxicity induced by BSO, AA, and Fe+AA (Bai and Cederbaum, 2001; Mari *et al.*, 2002; Wu and Cederbaum, 2002). If the decrease in mitochondrial membrane potential depends on the opening of the permeability transition pore, then a specific inhibitor should decrease the loss of mitochondrial membrane potential induced by the toxic agents. Cyclosporin A inhibited the loss of $\Delta\psi_m$ and the toxicity in CYP2E1-expressing cells exposed to AA, AA+Fe, and BSO (Caro and Cederbaum, 2002; Wu and Cederbaum, 2001, 2002), suggesting a role for the permeability transition on mitochondrial depolarization and subsequent toxicity. Additional evidence for increased mitochondrial damage in CYP2E1-overexpressing cells include the following: Depletion of GSH decreased oxygen uptake in permeabilized E47 cells with all respiratory substrates, and vitamin E prevented this decrease (Chen and Cederbaum, 1998). In CYP2E1-overexpressing cells treated with BSO + Fe-NTA, levels of ATP were lowered, and this was associated with a decreased rate of oxygen consumption by permeabilized cells with substrates donating electrons to complexes I, II, and IV of the respiratory chain. This mitochondrial damage was prevented by vitamin E (Sakurai and Cederbaum, 1998). Damage to mitochondria is an important event in the CYP2E1-dependent toxicity.

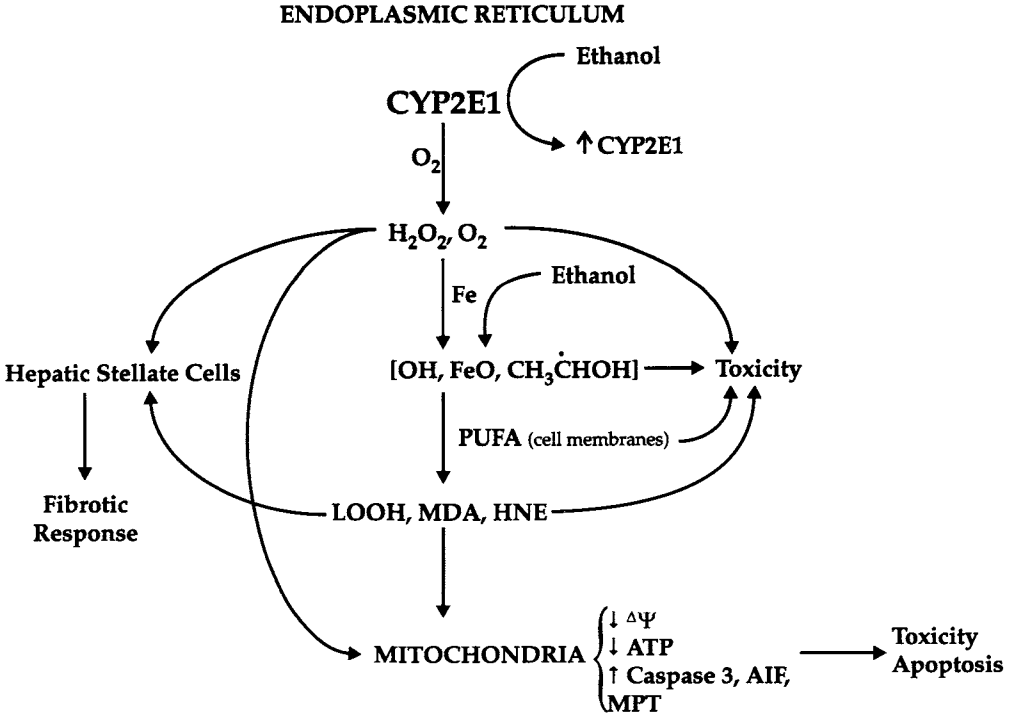

Fig. 2.1: A working model of CYP2E1-dependent oxidative stress and toxicity.