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Endothelin and hepatic wound healing

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Abstract

Liver wound healing is a coordinated response to injury caused by infections (hepatitis) or toxins (alcohol) or other processes where activation of hepatic stellate cells are a central component. During stellate cell activation, a major phenotypic transformation occurs which leads to increased production of increased extracellular matrix proteins and smooth muscle α-actin the results is organ dysfunction due to gross architectural disruption and impaired blood flow.

Endothelin-1 (ET-1) is produced in increased amounts and the cellular source of ET-1 shifts from endothelial cells to stellate cells during liver injury thus setting a feedback loop which accentuates further activation, stellate cell proliferation, and production of extracellular matrix proteins. Therapy directed at intervening the ET-1 signaling pathway has significant therapeutic potential in patients with liver disease.

Introduction

Liver wound healing is an orchestrated process characterized by fibrogenesis, remodeling, and gross distortion of liver architecture. Persistent and progressive accumulation of extracellular matrix proteins concurrent with a regenerative response gradually transforms the liver to a fibrotic, and cirrhotic structure.

Although a number of cell types have been advanced as important to the wounding response, hepatic stellate cells, which are retinoid rich, perisinusoidal cell of mesenchymal origin have a central role. After injury, this cell undergoes “activation”. The activation process is typical of all forms of liver injury and is characterized by morphological and functional features which include loss of retinoids, development of rough endoplasmic reticulum and acquisition of “smooth muscle-like” cytoskeleton, [1-3] (*Figure 1*). One of the major functional attributes of activated stellate cells is the production of extracellular matrix, including types I, III, IV collagens, fibronectin, proteoglycans in excessive amounts (*Figure 1)*; studies have shown that stellate cells from fibrotic animals exhibited a 30 to 40 fold increase in type I and type III collagen mRNA relative to normal stellate cells and

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hepatocytes respectively [4,5]. It has also been shown that stellate cells specifically acquire smooth muscle α actin during activation process (*Figure 1*), marking them as liver specific myofibroblasts [3,5,6].

A growing body of evidence has now suggested a crucial link between endothelin (ET) and the liver wound healing response; endothelin levels are elevated in diverse forms of injury and wound healing [7-10]. Abundant literature also suggests a critical role of ET in patients with liver cirrhosis.

Endothelin Biology

The family of ET's [11] are potent vasoconstrictor peptides made up of the three following peptides: ET-1, ET-2 and ET-3. Each peptide is a unique 21- amino acid residue that binds to G-protein coupled receptor (GPCR) including, the ET_A and ET_B [11,12]. Typically, ET is produced by endothelial cells and exerts paracrine effects on adjacent smooth muscle cells. However in pathological situations, ET appears to be synthesized in a broader range of cell types [9,13,14].

ET-1 synthesis is regulated at the level of synthesis of precursors as well as by their processing [15-17]. Pre-pro ET-1, the canonical precursor is induced by a variety of extracellular stimuli (e.g. shear stress) vasopressor hormones (e.g. vasopressin) and cytokines (e.g. interleukin-1) and a variety of vasoactive peptides (e.g epinephrine and angiotensin II) [18-20]. Proteolytic processing of prepro ET-1 by furin-like enzymes leads to production of intermediate ET, ultimately big ETs which are 38 to 40 amino acids residue (*Figure 2*). The big ETs have little or no biological effects and are cleaved at Trp-21- Val/ Ile-22 by specific endothelin-converting enzymes (ECE), to yield a the 21amino acid peptide which are biologically active [20] (*Figure 2*).

The ECE are neutral membrane-bound metalloproteases, a 120 kD endopeptidase-24 family of proteins, which belong to M13 group of proteins that includes neutral endopeptidases, kell blood group antigens (Kell), a peptide from phosphate regulating gene (PEX), X-converting enzyme (XCE), “secreted” endopeptidases, and the ECEs [21] found in brain [22-24]. Three isoforms of ECE have been reported [25], namely ECE-1, ECE-2 and ECE-3; ECE-1 and ECE-2 are most prominent [24]. ECE's. M13 family members contain type II integral membrane proteins with zinc metalloprotease activity [21], and their function is inhibited by phosphoramidon [24]. Four variants of ECE-1 have been reported in humans [26], namely ECE-1a, ECE-1b, ECE-1c and ECE-1d which are a result of alternate splicing of ECE-1 mRNA. ECE-1 appears to be localized to the plasma cell membrane and its optimal activity is at pH 7; it processes big ET's both intracellularly and on the cell surface [27]. It is distributed predominantly in smooth muscle cells. ECE-1 can also hydrolyze other proteins including bradykinin, substance P, and insulin [27]. ECE-2 is localized to the trans-Golgi network and is expressed abundantly in neural tissues and endothelial cells. Its optimal activity is at pH 5 [21,28]; the acidic activity marks ECE-2 as an intracellular enzyme [28]. Substrate selectivity experiments indicate that both ECE-1 and ECE-2 show preference for big ET-1 over big ET-2 or big ET-3 [29]. To date, evidence points to the existence of ECE isoforms or proteases other than ECE-1 and ECE-2 in the final ET processing step, since mice lacking both ECE-1 and ECE-2 produce mature ET-1 [30].

Some data suggest the presence of an ECE-1 independent pathway that process big endothelin, possibly involving tissue chymases and non-ECE metalloproteinases [31].

ET binds to two prominent G-coupled receptor subtypes, ET_A and ET_B which mediate a range of it's biologic effects [32]. ET_A receptors are predominantly expressed in vascular smooth muscle cells with the rank order affinities of the peptide as ET-1> ET-2 >> ET-3, while ET_B receptors are widely distributed and have equal affinity for all ET subtypes [12,33]. ET_A and ET_B receptors on smooth muscle cells mediate vasoconstriction while the ET_B receptor appears to mediate diverse responses depending on the cell type expressing the receptor. For example stimulation of ET_B receptors on endothelial cells stimulates Nitric oxide (NO) production and release and vascular smooth muscle relaxation. Interestingly, a variant of ET_B receptors also produces smooth muscle vasoconstriction [34].

The ET_A receptor is isoform selective and binds ET-1 and ET-2 with higher affinity than ET-3. In contrast, the ET_B receptor is not isoform specific and binds ET-1, ET-2 and ET-3 with equal affinity. In addition sarafatoxin (S6c), a peptide extracted from the venom of *Atractaspis engaddenensis* (Israeli burrowing asp) also serves as a ligand for ET_B receptor. Based primarily on functional assays [35]; two ET_B receptor subtypes have been proposed based on pharmacologic studies, ET_{B1} and ET_{B2}, however, the molecular basis for existence of these subtypes is still lacking [36]. The ET_C, a third type of ET receptor has been cloned from *Xenopus laevis and* displays a higher affinity for ET-3 than ET-1 and when stimulated causes release of NO [37].

ET signaling has been studied extensively (*Figure 3*). Upon binding to it's cognate receptors, ET leads to activation of phospholipase C [32], cleavage of phosphatidyl inositol 4, 5-biphosphate and subsequent release of second messengers inositol 1. 4, 5-triphosphate (IP3) and diacylglycerol (DAG) (*Figure 3*). The IP3 stimulates release of Ca^{2+} from endoplasmic reticulum leading to activation of Ca^{2+} calmodulin-dependant myosin light chain kinase, which in turn stimulates myosin Mg-ATPase and actomyosin cross bridging and contraction [38]. In addition other ET-1 stimulated signaling pathways have also been reported as for example: phosphatidyl choline-specific phospholipase (PC-PLD) and phosphatidic acid (PA) pathway in smooth muscle cells (*Figure 3*), ET-1 also stimulates protein tyrosine kinases (PTK) (*Figure 3*) such as RAF and RAS, particularly in neoplastic cells.

Endothelin and liver wound healing

Numerous lines of evidence support the importance of ET in the liver wound healing process. For example, circulating levels of ET-1 and ET-3 are increased in patients with cirrhosis, as well as in animal models of hepatic wound healing [7,8,39,40] and the source of ET in the injured liver is the organ itself [9,41]. Intrahepatic immunoreactive ET-1 levels have been found to correlate with the severity of liver disease [41]. Further, inhibition of ET signaling with ET receptor antagonists reduces the hepatic fibrogenic response [35,42-44].

These data emphasize the importance of ET in the hepatic wound healing response. Substantial effort has been directed at understanding both the signaling mechanisms underlying the effect of ET in the liver, as well as how ET (ET-1 in particular) is upregulated in injured liver. The mechanism underlying the increased production of ET in

the injured liver is complex, but is coming into better focus. In the normal liver, ET is produced primarily by sinusoidal endothelial cells; however, after injury, synthesis of ET-1 shifts from endothelial cells to hepatic stellate cells [9,45-47].

Another area of focus has been on the effect of ET-1 in the liver. A number of early studies demonstrated that the primary "target" of ET-1 in the liver is the hepatic stellate cell. [48-50]. This conclusion was drawn after it was shown that stellate cells possess abundant ET receptors, far in excess of other liver cells particularly after liver injury [48-50]. The study of ET receptors is of importance because ET receptors on hepatic stellate cells appear to have important biological effects- including proliferation, cell spreading, contractility, and even fibrogenesis- all features of the activated phenotype (see below). Interestingly ET receptor mRNA is unchanged or slightly decreased in stellate cells undergoing activation in culture or after carbon tetrachloride (CCl_4) induced injury. Further in one study, endothelin receptors appeared to be downregulated in stellate cells in response to TGF-β [51]. However, binding sites for ET-1 in stellate cells are markedly increased after their activation [48]. Additionally, ET_B receptors were upregulated in stellate cells after their activation [52]. This is in contrast with other work, in which it has been shown that there is dramatic upregulation of ET receptors in rat livers after endotoxin treatment [53]. The reason for the discrepancy in reports of endothelin receptors expression in stellate cells is unclear and may be related to divergent culture conditions or different biologic systems.

Any discussion of the role of stellate cells in wound healing requires an understanding of hepatic stellate cell "activation". This process, which essentially involves transdifferentiation of the cell from a quiescent state to an activated, myofibroblast-like cell is associated with loss of vitamin A droplets, increased rough endoplasmic reticulum, dynamic cytoskeletal changes (i.e. such as expression of smooth muscle α-actin, and fibrogenesis [54,55]. Activation of hepatic stellate cells appears to be a key step in the development of liver cirrhosis and fibrosis. Activated stellate cells have many functional roles, prominent among which are the production of pathological amounts of extracellular matrix (ECM) proteins [56-58]. These cells also proliferate and secrete a variety of cytokines that not only stimulate stellate cells in an autocrine manner, but also are important in perpetuating the entire wounding response [59]. Additionally, the acquisition of a robust actin based cytoskeleton in activated stellate cells imparts a contractile phenotype and appears to be directly correlated with force generation and regulation of sinusoidal blood flow which is disturbed in portal hypertension [42,60].

Stellate cell activation is stimulated by several important factors in the wounding environment including growth factors, cytokines, chemokines, oxidative stress, and the ECM itself [61]. ET-1 is one of the wide array of factors that appear to contribute to stellate cell activation [35]. ET-1 directly stimulates expression of smooth muscle α-actin in cultured stellate cells [62]. Further, the ET receptor antagonist, bosentan, inhibited fibrogenesis in a chronic liver injury model, consistent with an important role for ET-1 in wound healing [35].

Available data further suggest complex interplay between TGF-β and ET-1 in modulating hepatic stellate cell activation in each a paracrine and autocrine manner [45]. ET-1 has been

found to increase TGF-β1 mRNA and also stimulates release of TGF-β1 in stellate cells [63]. The autocrine functional effects of ET-1 in stellate cell activation have been emphasized in a signaling loop that involves a fibronectin-Shc-SRC-ERK dependant mechanism [47]. In liver injury, it has also been shown that TGF-β dependent ET-1 signaling proceed through differential signaling pathways depending on the mechanism of liver injury that leads to stellate cell activation [64]. For example, in one study, in CCl_4 induced injury, TGF-β dependent ET-1 signaling proceeded through a p38 MAPK pathway [64], while after bile duct ligation, signaling was mediated through a TGF-β dependant extracellular regulated kinases (ERK) pathway [64]. Although the precise mechanism of interaction between ET-1 and TGF-β has not been elucidated in stellate cells, it may be a result of cross talk between the ET-1 and TGF-β receptor as has been demonstrated in vascular smooth muscle cells [65].

One of the most prominent phenotypes in stellate cells is their production of extracellular matrix [66] which has been shown to be stimulated by ET-1, the latter of which appears to be at least partially TGF-β dependant [45]. Additionally, other ET-1 induced phenotypes like such as keratinocyte transdifferentiation and contractility [67,68] also appear to be TGF-β dependant. ET-1 may interact with other cytokines during liver injury, although this area is less well studied than TGF-β, some data suggest cross talk between ET-1 and PDGF in liver wound healing [69]. PDGF mediates chemotactic recruitment, and early proliferative responses of hepatic stellate cells in liver fibrosis [70,71]. However, the precise mechanism of interaction between ET-1 and PDGF has not been fully determined.

One of the most prominent roles of ET-1 in liver wound healing is to induce cellular contraction. The pathways by which ETs induce cellular contraction appears to be complex. The canonical signaling pathway by which ET-1 induces cellular contraction in smooth muscle cells is via Ca^{2+} dependant signaling [72] (*Figure 3*). In this pathway, stimulation of ET_A receptors leads to activation of phospholipase C and formation of inositol 1, 4, 5 triphosphate (IP_3) and diacylglycerol (DAG) from phosphatidyl inositol [32]. Further, IP_3 interacts with specific receptors localized in the endoplasmic reticulum, releasing Ca^{2+} from Ca^{2+} stores into the cytosol [32,72,73] (*Figure 3*). A rapid elevation cytosolic Ca^{2+} then activates the Ca^{2+}/calmodulin dependant myosin light chain kinase (MLCK) which in turn leads to increased phosphorylation of regulatory myosin light chain (rMLC). Phosphorylation of rMLC leads to activation of myosin ATPase, actomyosin bridging and cellular contraction [74,75]. Evidence suggests that stellate cells employ Ca^{2+} dependent contraction [76,77], which may be enhanced after activation [78].

A unique attribute of ET-1 induced contraction, described in particular for non-smooth muscle cells, including activated hepatic stellate cells (myofibroblasts) appears to be via a Ca^{2+}independent pathway, thought to be activated via protein kinase C (PKC) [77], integrin-linked kinase (ILK) ([79,80], Raf-1 [81] and Rho-associated kinase (ROCK) [82]. PKC and ROCK have been associated with reduced activity of myosin light chain phosphatase (MLCP) and prolonged activation of rMLC, and thus contraction [83]. In stellate cells, ET-1 is capable of inducing cellular contractility independent of Ca^{2+}, in addition to more classical contraction pathways [77,80,84].

A further important point is that activated stellate cells (i.e., myofibroblasts) have been shown to exhibit an enhanced contractile phenotype after liver injury [35,85]. This has important implications for disease in the liver, via several putative mechanisms. First, stellate cells are known to reside in a perisinusoidal fashion [86], akin to pericytes in the periphery; pericytes appear to regulate blood flow. Thus, it has been proposed that stellate cells function as liver specific pericytes, and regulate sinusoidal blood flow [87-89]. This function appears to be important in regulation of intrahepatic resistance, and further may contribute to increased resistance in portal hypertension [87]. Interestingly, the degree of increase in contractility appears to be proportional to the expression of the putative contractile protein, smooth muscle α-actin [35].

Stellate cell activation is also characterized by fibrogenesis – i.e., by the production of increased quantities of ECM proteins including types I, III, and IV collagens, fibronectin, laminin and proteoglycans [4] with type I collagen constituting a high proportion in liver fibrosis [90]. Abundant evidence indicates that activated hepatic stellate cells are a major source of ECM proteins produced during fibrogenesis [91]. Activation is also associated with dysregulation of matrix-degrading enzymes matrix-metalloproteinases (MMP) and their inhibitors (such as tissue inhibitors of metalloproteinases (TIMPs) [63]. For example, MMPs may have suppressed activity in the injured liver due to increased expression of TIMPs [63] indicating that ECM deposition occurs as a result of excessive production as well as decreased degradation. Indeed, it appears that the ECM plays a dynamic role in modulating the wound healing events through it's continuous synthesis and degradation [92,93] thus providing scaffold for tissue construction as well as deterioration of vital function of the organ [94].

A large body of experimental literature suggests a relationship between ET-1 and fibrogenesis (*Figure 1*). It has been shown that ET receptor antagonists inhibit liver fibrosis [35,43,95-97]. For example, bosentan, a mixed ET receptor antagonist used in rat liver fibrosis suppressed the activation of stellate cells and reduced levels of type I collagen synthesis [35]. An ET_A receptor antagonist also reduced fibrosis [95]. TAK-044, an ETA receptor antagonist treatment reduced collagen synthesis, as evidenced by decreased hepatic hydroxyproline content, mRNA expression of collagen-alpha type I, and tissue inhibitors of matrix metalloproteinases 1 and 2, and mRNA and protein expression of a TGF-β in CCl_4 and LPS induced cirrhosis in rats, respectively [96] [98].

The mechanism for amelioration of hepatic fibrogenesis has been suggested to be via direct effects on the fibrogenic cascade in stellate cells as well as by interplay with other fibrogenic mediators such as TGF-β [99], platelet derived growth factor (PDGF) [69,71] and tumour necrosis factor (TNF-α) [100] (*Figure 1*) and interferon gamma (Li, T- abstract- AASLD 2008).

Another important role of ET-1 in the wounding environment appears to be cell proliferation. ET-1 is known to be important in smooth muscle cell proliferation, as well as in neoplastic cells [101-103]. Although, proliferative effects of ET-1 have been reported on quiescent stellate cells [9,104] analogous to other cell types [101,102], It's proliferative effects on cells *in vivo* situation is controversial [66,105,106]. For example, studies on

human hepatic stellate cell, derived from outgrowth of normal-liver explant tissue, have shown that ET-1 had mitogenic effects primarily on quiescent cells, yet it had anti-proliferative effects on activated cells [66,105]. The mechanism of this anti-proliferative effect appears to be mediated through ET_B receptors, which are prominent in activated cells [66]. It has been suggested that NF-kappaB and cyclooxygenase-2 (COX-2) [107] signaling through ET_B receptors may facilitate the anti-proliferative effects through a cAMP mechanism [108]. The mechanism underlying the discrepancy in ETs effect on quiescent (proliferative) and activated cells (anti-proliferative) remains unknown, but may be related to culture conditions under which stellate cells were grown. Further, different proportions and effects of ET_A and ET_B receptors in these cells at different stages of myofibroblastic differentiation may determine the proliferative response of ET-1 in stellate cells.

Although beyond the scope of this review, ET-1 appears to be important on other forms of wound healing, including in the heart, lung, skin, kidney, and vasculature [109-113].

Future

Given the apparent benefit of ET receptor antagonists in liver wound healing, it is possible that these could be effective in humans with fibrosis. Currently, very few human studies are available. ET_A antagonist, BQ-123 and ET_B antagonist, BQ-788 were studied in patients with a history of variceal bleeding [114], and did not show any effect on hepatic vein pressure gradient [114]. Another study suggested that an ET_A endothelin antagonist might improve vascular tone in patients with cirrhosis [115]. Studies published to date have been performed with smaller number of patients and perhaps the use of sub-therapeutic doses of ET receptor antagonists. A further impediment to development of clinical trials has been concern about drug induced hepatototoxicity; fatal acute hepatitis and liver injury in patients receiving ET receptor antagonists has been reported and thus has led most clinicians to avoid the use of ET receptor antagonists in patients with liver disease [116,117]. ET receptor antagonists studied in clinical trials for different conditions are highlighted in Table I. Although there is concern about toxicity of ET receptor antagonists in patients with cirrhosis, the preclinical data suggest that there is significant translational potential for antagonism of the endothelin system in patients with cirrhosis.

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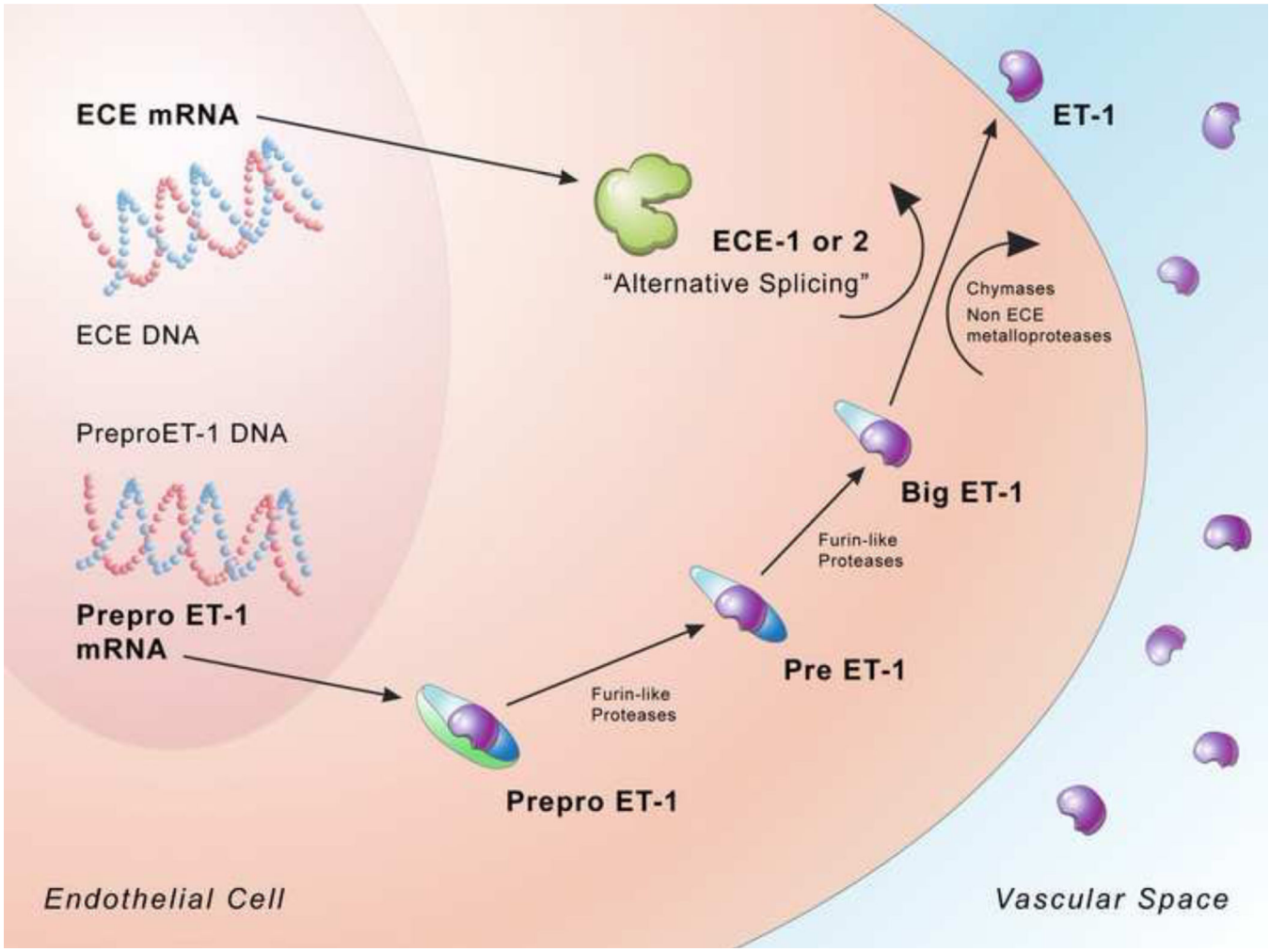

Figure 1. The role of ET-1 in stellate cell activation and hepatic fibrogenesis

Stellate cell activation is characterized by typical phenotypic features including the loss of retinoids, proliferation, development of a robust rough endoplasmic reticulum and acquisition of a “smooth muscle-like” cytoskeleton. ET-1 is one of a wide array of factors that appear to contribute to stellate cell activation and directly stimulates expression of smooth muscle α-actin. On the left side of the image, various cytokines stimulate ET-1 synthesis in activated stellate cells, ET-1 then has autocrine effects on stellate cells themselves, which fuel the fibrogenic cascade to stimulate synthesis of ECM proteins. The ultimate result of the wounding response is fibrosis, and in the liver, cirrhosis with complications that include portal hypertension, hepatocellular dysfunction, and even hepatocellular cancer. Abbreviations: TGF-β = transforming growth factor-β; TNF-α = tumor necrosis factor-α, PDGF = platelet- derived growth factor

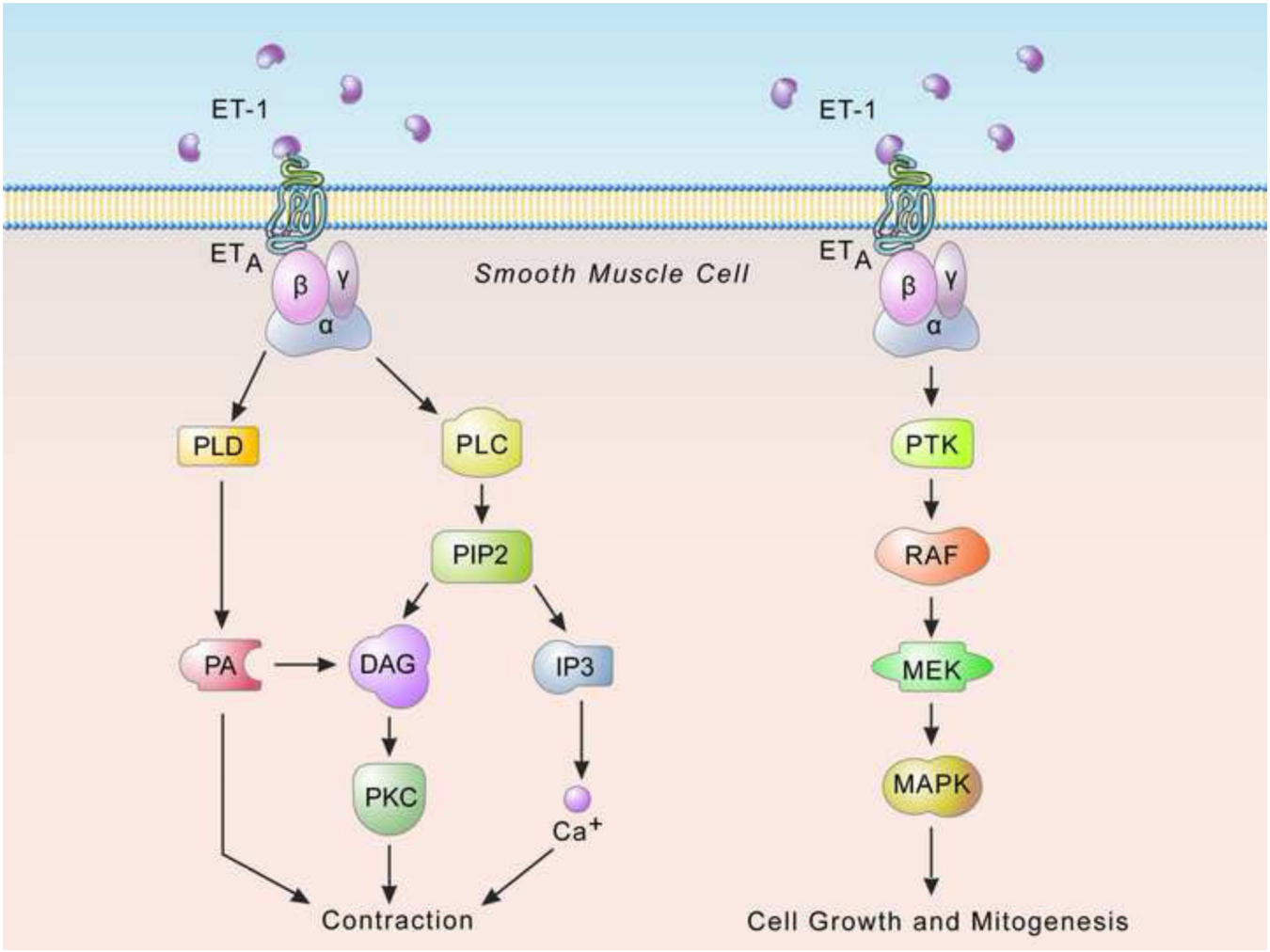

Figure 2. Endothelin-1 biosynthetic pathway

Pre-pro ET-1, the canonical precursor is synthesized as a product of transcription from preproendothelin , a 203-amino acid peptide that cleaved at dibasic sites by furin-like endopeptidases to form intermediate ETs, and then big ET's which are 38 to 40 amino acid residues. The intermediate and big ET's have no biological activity but undergo cleavage at Trp-21- Val/Ile-22 by specific endothelin-converting enzymes (ECE), to yield a the mature 21aa ET-1 which is biologically active (from Khimji AK and Rockey DC. Endothelin-biology and disease. *Cell Signal* 2010 11: 1615 with permission).

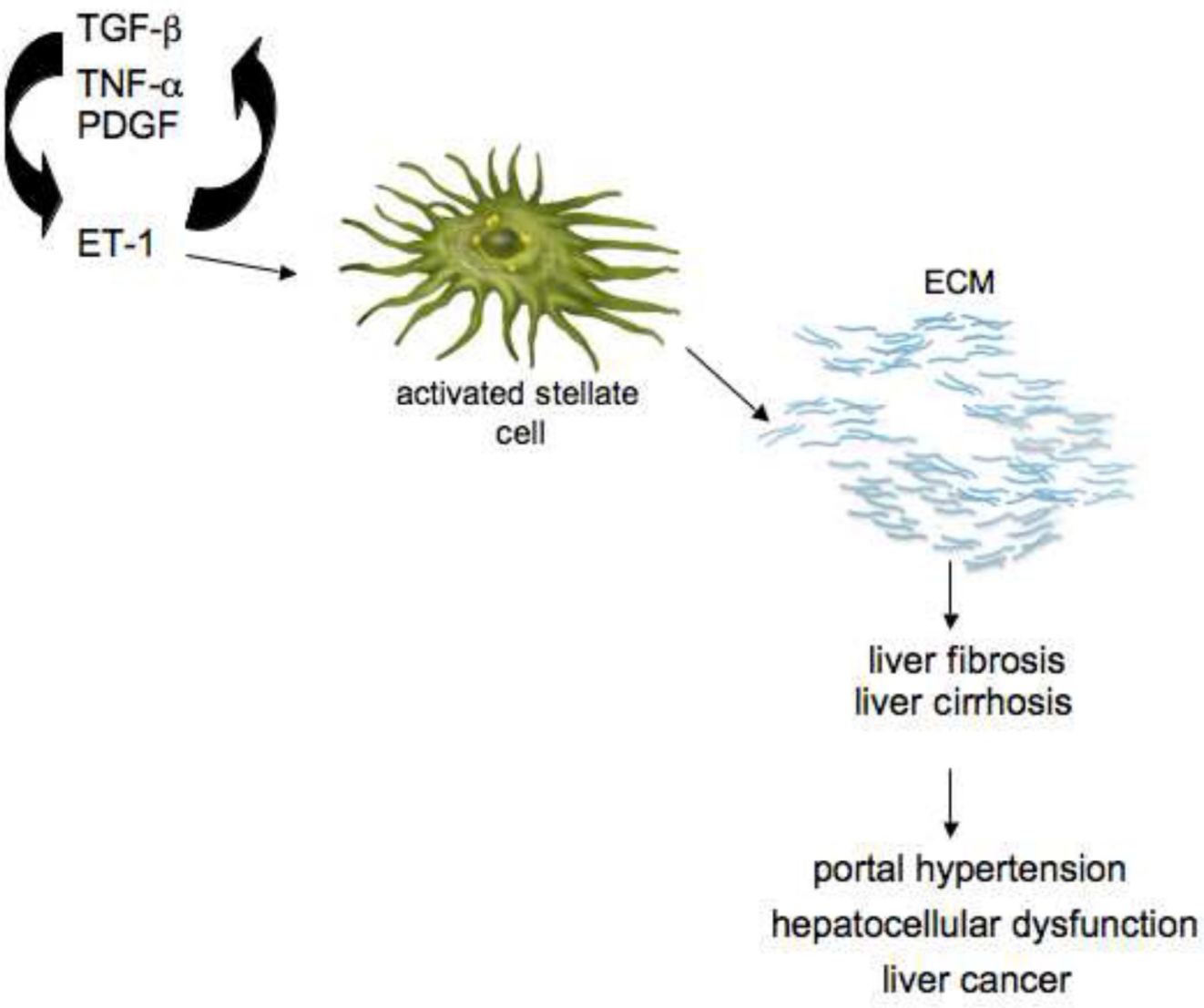

Figure 3. Endothelin signaling

ET binds to two prominent G-coupled receptor subtypes, ET_A and ET_B. ET receptor stimulation is followed by activation of a variety of different downstream cascades. For example, shown on the left, ET_A induced activation of phosphatidyl inositol specific phospholipase C (PI-PLC) leads to the formation of inositol triphosphate and diacylglcerol (DAG) from phosphatidylinositol. Inositol 1, 4, 5 triphosphate (IP_3) which then diffuses to specific endoplasmic reticulum receptors and releases stored Ca^{2+} into the cytosol. This causes a rapid elevation in intracellular Ca^{2+} which in turn causes cellular contraction. In addition, ET_A stimulation also induces activation of phosphatidyl choline-specific phospholipase (PC-PLD) yielding phosphatidic acid (PA) (shown on the left), PA is dephosphorylated by PA phosphorylase to DAG and DAG is also metabolized to PA by DAG kinase. ET-1 also stimulates protein tyrosine kinases (PTK) (shown on the right) such as RAS, particularly in neoplastic cells. Activation of PTKs in this pathway results in induction of the RAF/MEK/MAPK pathway, subsequently stimulating transcription of

protooncogenes such as c-FOS, c-MYC, c-JUN, which in turn activate cell growth and metastasis (from Khimji AK and Rockey DC. Endothelin-biology and disease. *Cell Signal* 2010 11: 1615 with permission).

Table 1
current status of ET receptor antagonists

Use of ET receptor antagonists for pulmonary hypertension has become well established. Animal models suggest benefit in other disorders, and they have been studied in those highlighted; however, proof of their effectiveness at this point is generally modest.

Compound	ET receptor target	Clinical trial disease	Reference
Bosentan	ETA/B	pulmonary hypertension	[118]
		congestive heart failure	[118]
		coronary artery disease	[119]
		scleroderma	[120]
		cerebral vasospasm	[118]
Sitaxsentan	ETA	pulmonary hypertension	[116]
		chronic heart failure	[121]
Ambrisentan	ETA	pulmonary hypertension	[122]
		chronic heart failure	[121]
Atrsentan	ETA	prostate cancer	[123]
S-0139	ETA	cerebrovascular ischaemia	[124]
BQ-123	ETA	cirrhosis and portal hypertension	[114]
BQ-788	ETB	cirrhosis and portal hypertension	[114]