

REVIEW

Hepatic stellate cells: role in microcirculation and pathophysiology of portal hypertension

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Gut 2002;50:571–581

Accumulating evidence suggests that stellate cells are involved in the regulation of the liver microcirculation and portal hypertension. Activated hepatic stellate cells have the necessary machinery to contract or relax in response to a number of vasoactive substances. Because stellate cells play a role in both fibrosis and portal hypertension, they are currently regarded as therapeutic targets to prevent and treat the complications of chronic liver disease.

SUMMARY

Portal hypertension is the result of augmented intrahepatic vascular resistance and increased portal blood flow. For many years it has been accepted that hepatic stellate cells play a key role in hepatic fibrosis. Accumulating evidence from *in vitro* and *in vivo* studies suggests that stellate cells are also involved in the regulation of the liver microcirculation and portal hypertension. Activated hepatic stellate cells have the necessary machinery to contract or relax in response to a number of vasoactive substances. Although contractile mechanisms are not as well studied as in skeletal or smooth muscle cells, recent studies have demonstrated that some intracellular signalling pathways and proteins involved in muscle contraction (for example, intracellular Ca^{2+} or rho) also exist in hepatic stellate cells. Because stellate cells play a role in both fibrosis and portal hypertension, they are currently regarded as therapeutic targets to prevent and treat the complications of chronic liver disease.

INTRODUCTION

According to Ohm's law ($\Delta P=Q \times R$), portal venous pressure is proportional to blood flow and resistance: ΔP is the change in portal pressure along the vessel, Q is portal blood flow, and R is the resistance to flow.¹ In the normal liver, intrahepatic resistance changes with variations in portal blood flow, thereby keeping portal pressure within normal limits. In hepatic cirrhosis however, intrahepatic resistance and splanchnic blood flow are increased. Portal hypertension is thus the consequence of a combination of decreased compliance and increased portal blood flow.

The initial event in the pathophysiology of portal hypertension is increased vascular resistance to portal flow, primarily caused by structural changes such as fibrotic scar tissue and regenerative nodules compressing portal and central venules. Furthermore, it has been shown that

swelling of hepatocytes and capillarisation of hepatic sinusoids (loss of endothelial fenestrations and collagen deposition in the space of Disse) are part of the increased vascular resistance. Increased blood flow in the portal vein occurs in a more advanced stage of portal hypertension and contributes to its maintenance and aggravation. Increase in portal inflow results from hyperdynamic circulation in the splanchnic and systemic blood vessels, which is characterised by increased cardiac output, reduced mean arterial pressure, reduced systemic vascular resistance, and expanded plasma volume. Although structural changes are most important in intrahepatic vascular resistance, it has become clear in the past years that not only fixed, but also variable, factors contribute significantly to the increased hepatic vascular resistance.² It has been demonstrated that intrahepatic vascular resistance can be reduced by 20–30% with pharmacological agents.^{2,3} Several ultrastructural and physiological features of hepatic stellate cells (HSC) are similar to pericytes in other organs, suggesting that HSC may function as liver specific pericytes. Indeed, both the anatomical location of HSC and the capacity to contract or relax in response to various vasoactive mediators suggest that these cells may play a role in modulating intrahepatic vascular resistance and blood flow at the sinusoidal level.^{4–6} Several investigators have shown that activated HSC contract in response to various agents of which endothelin-1 (ET-1) is the strongest, and that some agents—for example, nitric oxide (NO)—promote relaxation of HSC. Moreover, HSC play a key role in the development of hepatic fibrosis, the main cause of portal hypertension.⁷ The aim of this review is to summarise data on HSC contractility, their potential role in the liver microcirculation, and the pathophysiology of portal hypertension.

HEPATIC STELLATE CELLS

HSC represent 5–8% of all human liver cells and 13% of the volume of sinusoidal cells. Stellate cells

Abbreviations: ANP, atrial natriuretic peptide; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; $[Ca^{2+}]_i$, intracellular Ca^{2+} ; DAG, diacylglycerol; ET-1, endothelin-1; $ET_{A/B}$, endothelin receptor A/B; FAK, focal adhesion kinase; cGMP, cyclic guanosine monophosphate; HO, haeme oxygenase; HSC, hepatic stellate cells; IP3, inositol 1,4,5-trisphosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid; NO, nitric oxide; NOS, nitric oxide synthetase; MLC, myosin light chain; PI(3)K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PLD, phospholipase D; PKC, protein kinase C; ANGII, angiotensin II; AT1, type 1 angiotensin receptor.

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Accepted for publication 17 July 2001

are located in the perisinusoidal space of Disse beneath the endothelial barrier. They have long cytoplasmic processes which run parallel to the sinusoidal endothelial wall. Second order branches sprout out from the processes, embrace the sinusoid, and penetrate between hepatocytes reaching neighbouring sinusoids. Some HSC are in close contact with nerve endings, some of which contain neuropeptides such as substance P, neuropeptide Y, somatostatin, and calcitonin gene related peptide.^{8,9}

“Hepatic stellate cells represent 5–8% of all human liver cells”

In normal liver, HSC are mainly involved in the storage of vitamin A. In addition, they synthesise extracellular matrix components, matrix degrading metalloproteinases, cytokines, and growth factors.¹⁰ Following acute or chronic liver injury, HSC are activated and undergo a process of transdifferentiation leading to a myofibroblastic phenotype. Activated cells are characterised by loss of vitamin A droplets, increased proliferation, release of proinflammatory, profibrogenic, and prometogenic cytokines, migration to sites of injury, increased production of extracellular matrix components, and alterations in matrix protease activity providing the fundamental needs for tissue repair. In acute or self limited liver damage, these changes are transient whereas in the case of persistent injury they lead to chronic inflammation and accumulation of extracellular matrix resulting in liver fibrosis and ultimately cirrhosis. Several growth factors and cytokines are involved in HSC activation and proliferation, of which transforming growth factor β and platelet derived growth factor are probably the most important.⁷

Furthermore, activated HSC express the smooth muscle α actin gene. In the branching processes, α smooth muscle actin and desmin filaments are present. Both the three dimensional structure and some of the ultrastructural characteristics are similar to pericytes, regulating blood flow in other organs. Hence HSC are currently considered to exert pericyte functions in the hepatic sinusoid.

ROLE OF HEPATIC STELLATE CELLS IN REGULATING BLOOD FLOW IN NORMAL LIVER

The liver is a richly perfused organ receiving approximately 25% of the cardiac output. About 75% of hepatic blood flow (rich in nutrients but poorly oxygenated) is supplied by the portal vein. The remainder of the blood supply (oxygen rich) is provided by the hepatic artery. The intrahepatic vasculature is composed of portal venules, hepatic arterioles, lymphatics, hepatic sinusoids, and central venules. Most blood enters the sinusoids from portal venules through inlet sphincters. Branches of hepatic arterioles terminate in sinusoids near their origin from portal venules (arterio-sinus twigs).

“Data indicate that HSC may play a role in blood flow regulation in the normal liver”

Furthermore, intersinusoidal sinusoids connect neighbouring sinusoids and finally sinusoids receive blood from arteriportal anastomoses via portal venules. Sinusoids drain their blood in central venules through outlet sphincters.^{11,12} The hepatic sinusoid, which is the principal site of blood flow regulation, is the narrowest vascular structure within the liver; the highest vascular resistance occurs in the sinusoids.¹³ Moreover, it is the vital site for transvascular exchange between blood and hepatocytes. The sinusoidal surface of the hepatocytes is separated from blood by fenestrated sinusoidal endothelial cells lining the sinusoid, Kupffer cells (liver macrophages) protruding into the lumen of the sinusoid, Pit cells (liver specific natural killer cells) and HSC, also referred to as

Ito cells, lipocytes, fat storing cells, or hepatic perisinusoidal cells.⁸ From the above it is clear that there are several potential sites for sinusoidal blood flow regulation: (i) portal venules, (ii) hepatic arterioles, (iii) central venules, and (iv) sinusoids, with inlet sphincters, outlet sphincters, and sinusoidal cells of which HSC and sinusoidal endothelial cells have contractile properties. Data indicate that HSC may play a role in blood flow regulation in the normal liver.

The first direct evidence was provided by in vivo microscopy in normal rat liver.^{14–18} Infusion of ET-1 in the portal vein caused a marked decrease in sinusoidal diameter and an increase in sinusoidal pressure gradient and resistance. ET-1 was found to induce significant sinusoidal constriction at the sites of HSC but not at the sites of Kupffer cells or sinusoidal endothelial cells. Coadministration of L-NAME and an endothelin receptor B (ET_B) agonist resulted in significant bulging of HSC into the sinusoidal lumen and reorganisation of fat droplets in the cell body in a way consistent with shape changes accompanying cell contraction.¹⁹ Recently, some evidence was obtained that locally produced carbon monoxide might control sinusoidal blood flow in normal liver. Carbon monoxide is produced by degradation of haeme, mediated by the activity of haeme oxygenase (HO), and upregulates cyclic guanosine monophosphate (cGMP) thereby causing smooth muscle relaxation. Three isoforms of HO have been described: (i) HO-1, the inducible form (ii) HO-2, the constitutive form, and (iii) HO-3, a form with a low catalytic activity.²⁰ Isoforms HO-1 and HO-2 are expressed in the liver: HO-1 in Kupffer cells and HO-2 in hepatocytes.²¹ Inhibition of endogenously produced carbon monoxide promoted sinusoidal contraction at sites that colocalised with HSC, suggesting that these cells may be the target of the relaxing effect of carbon monoxide.²² Even though these observations are suggestive, they do not prove that HSC modulate hepatic blood flow. Indeed, it has been shown that ET-1 induced presinusoidal sphincter constriction resulting in decreased sinusoidal blood flow without stellate cell contraction.²³ This raises the question of whether sinusoidal diameter narrowing is not related to passive recoil of sinusoids secondary to decreased sinusoidal blood flow. In fact, experimental clamping of the portal vein has been shown to result in passive recoil of sinusoids and portal venules.²⁴ Discrepant results are probably related to different doses of ET-1 and a different morphological method of assessing vessel diameter after tissue fixation of isolated perfused livers. ET-1 probably has sinusoidal as well as extrasinusoidal sites of action but the sinusoidal sites appeared to be sensitive to lower more physiological concentrations of ET-1.¹⁷

In vitro experiments are also controversial. It has been demonstrated that HSC, cultured for 24 hours, relaxed in response to interleukin 1²⁵ and contracted in response to substance P and ET-1.^{26,27} Higher concentrations of ET-1 were required to obtain a significant increase in intracellular Ca²⁺ ([Ca²⁺]_i) and contraction of HSC cultured for one day than of activated HSC.²⁷ However, these observations could not be confirmed by other investigators.^{28,29} Possible explanations for discrepant findings include damaging of receptors by isolation of cells and differences in the set up of contraction experiments. Indeed, although quiescent HSC may not possess the necessary machinery required for forceful contraction, weaker contractions might occur. To show collagen lattice contraction, a more forceful contraction of cells is required than the force needed to demonstrate contraction of isolated cells using phase contrast microscopy. Arguments against contraction of HSC in normal liver include: (i) HSC do not have a stellate shape but rather a spider-like shape which is unlikely to be contraction ready, (ii) effective contraction is limited by spatial constraint in the space of Disse and contraction would be possible only by cells with a powerful contractile machinery, and (iii) the presence of lipid droplets prevents microfilament assembly into a long span.³⁰ Taken together, although some arguments are against,

most evidence suggests that HSC are involved in the regulation of sinusoidal tone and blood flow in normal liver.

CONTRACTILE APPARATUS OF ACTIVATED STELLATE CELLS.

Actin microfilaments are a major component of the skeletal framework that determines cell shape. In addition, actin and myosin filaments form together the contractile apparatus of cells. In each case, in addition to the major proteins involved, there are many other proteins whose binding modulates the behaviour of these cell components. It must be stated that the information regarding the proteins involved in these complexes in stellate cells is negligible and that only assumptions can be made by analogy with smooth muscle cells which have some resemblance to activated stellate cells.

The components of all contractile apparatus are the thin actin filaments and the thick myosin filaments. Actin filaments are formed by polymerisation of actin monomers on a tropomyosin backbone. The actin filament lengths are determined by attachment of "capping" proteins such as tropomodulin which in fact bind to tropomyosin and prevent further extension. In muscle there are cytoskeletal and contractile actin filaments that are distinguished by different isoforms of both tropomyosin and actin.

The key to contraction is the shielding and unshielding of the myosin binding site on actin which at rest is blocked by tropomyosin (fig 1). The process is well understood in skeletal muscle where troponin binds to tropomyosin to modulate its position on the actin filament. Calcium binding to troponin causes movement of tropomyosin to expose the myosin binding site on actin to which myosin then binds, activating the actomyosin ATPase and giving rise to muscle contraction.³¹ The mechanism in smooth muscle is much less clear. It appears that the same isoforms of actin and tropomyosin are involved in the contractile elements of smooth muscle. Hence it is reasonable to suppose that tropomyosin will block the myosin binding site on actin as in skeletal muscle. This leads to the expectation that a mechanism for moving tropomyosin to expose the myosin binding site must also exist in smooth muscle.

In smooth muscle, phosphorylation of the regulatory light chain subunit of myosin is sufficient for contraction. However, contraction can occur without light chain phosphorylation. There is no simple relationship between the extent of phosphorylation and the force developed. Smooth muscle can remain tonically contracted with very little energy expenditure with a reduced rate of cross bridge cycling and ATP hydrolysis.³² It appears that a small number of high affinity myosin-actin cross bridges, possibly phosphorylated, switch actin filaments into a state that activates cross bridge cycling. The binding of each high affinity cross bridge switches adjacent actin monomers causing additional cross bridges. Tropomyosin enhances this effect by increasing the number of actin monomers that switch as a unit. Therefore, actin filaments and their associated binding proteins, tropomyosin, caldesmon, and calponin, have a major role in the contraction process in smooth muscle.

Tropomyosins have two major variants: short forms of 248 amino acids that are found in cytoskeletal actin filaments and long forms of 284 amino acids that are found in contractile actin filaments of muscle. There is great potential for isoform variation by variable splicing of the tropomyosin genes. The secondary and tertiary structure of the proteins is highly conserved (even from yeast to mammals) but there is potential for important functional changes from the splicing of alternative exons available in each of the genes. This is likely to affect binding to actin and the binding of regulatory proteins to tropomyosin.

The two main candidate proteins for the important role of modifying the tropomyosin binding conformation with actin

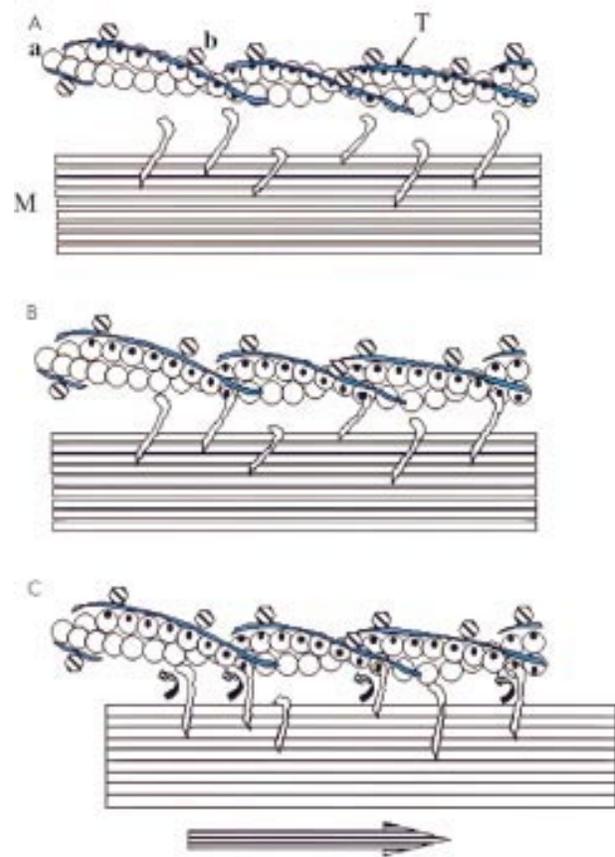


Figure 1 (A) Thin actin filament (a) and thick myosin filament (M) at rest, with tropomyosin (T) covering the myosin binding site and binding proteins with tropomyosin (b). In skeletal muscle, b is troponin; in smooth muscle it is likely to be calponin or caldesmon. (B) Activation of actomyosin: tropomyosin moves from myosin binding sites on actin in two stages—in skeletal muscle, stage 1 is caused by calcium binding to troponin while in smooth muscle stage 1 can be caused by myosin phosphorylation but that is not obligatory. In both cases initial myosin binding causes cooperative conformational changes in stage 2 to fully expose the actin binding sites for myosin. (C) Resulting actomyosin ATPase gives energy for a conformational change in myosin that moves the thick filament relative to the actin thin filament.

molecules in thin filaments are caldesmon and calponin.³³ Caldesmon has two isoforms and the higher molecular weight variant of 150 kDa is found associated with tropomyosin in actin filaments of smooth muscle. In the presence of tropomyosin, caldesmon can inhibit actomyosin ATPase activity. This ability is modulated by phosphorylation of caldesmon. Differentiated smooth muscle cells show transcriptional upregulation of caldesmon and tropomyosin. In addition, isoform changes in caldesmon and tropomyosin by alternative splicing are completely coordinated with the phenotype of smooth muscle cells.³⁴ The site for calcium dependent regulation of actin-myosin interaction resides in the COOH terminal domain of both the low and high molecular weight forms. However, the high molecular weight form is expressed in differentiated smooth muscle cells whereas this is replaced by the low molecular weight isoform during dedifferentiation.

Three isoforms of the tropomyosin and actin binding protein calponin have been identified. Neutral calponin is found in the cytoskeleton bound to tropomyosin in actin filaments.³⁵ Basic calponin is found in the actin filaments of the contractile elements of smooth muscle and has been used as a marker of smooth muscle phenotype. Cells expressing neutral calponin spread more efficiently than those expressing basic calponin and the latter also show reduced cell motility in

Table 1 Agents influencing hepatic stellate cell contractility

Agent	Effect	Proposed mechanism
Endothelin-1	Contraction	[Ca ²⁺] _i
Thrombin	Contraction	[Ca ²⁺] _i
Angiotensin II	Contraction	[Ca ²⁺] _i
Vasopressin	Contraction	[Ca ²⁺] _i
Adenosine	Contraction	[Ca ²⁺] _i ?
Substance P	Contraction	[Ca ²⁺] _i
Leukotriene D ₄	Contraction	[Ca ²⁺] _i
PGF ₂ /thromboxane	Contraction	[Ca ²⁺] _i
Lysophosphatidic acid	Contraction	Rho kinase
NO	Relaxation	cGMP
ANP	Relaxation	cGMP/[Ca ²⁺] _i
Adrenomedullin	Relaxation	cAMP
Somatostatin	Relaxation	[Ca ²⁺] _i /rho kinase?
Agents increasing cAMP/cGMP	Relaxation	cAMP/cGMP
PGI ₂ /PGE ₂	Relaxation	cAMP
Y-27632 (rho kinase inhibitor)	Relaxation	Rho kinase

PG, prostaglandin; NO, nitric oxide; ANP, atrial natriuretic peptide.

wound healing. Acidic calponin is particularly expressed in neurite outgrowths. So far, acidic calponin has not been demonstrated in smooth muscle cells.³⁵ Calponin inhibits actomyosin ATPase activity and actin filament movement in vitro. Calponin inhibition of the actin-myosin interaction can be blocked by protein kinase C (PKC) mediated phosphorylation of serine-175.³⁶

Although protein kinases have a role in cell signalling related to actin filaments, the final signalling pathway to the controlling actin filament proteins remains unclear. Furthermore, expression of the proteins discussed above is unknown in stellate cells together with how such factors as isoform expression vary during activation. Different isoform expression can be predicted to have a major effect on their behaviour.

CONTRACTILITY OF HEPATIC STELLATE CELLS: INTRACELLULAR SIGNALLING

Recent years have seen considerable progress towards understanding the signalling mechanisms regulating contraction in both muscle and non-muscle cell types. Studies to date have elucidated two major pathways: (i) Ca²⁺ signalling, which appears to predominate in smooth muscle, and (ii) the low molecular weight GTPase, rho pathway, which seems to exert the major controlling influence in non-muscle cells.^{37,38}

"To date, a number of agents which elicit either HSC contraction or relaxation have been identified"

At present, it is not clear which of these pathways predominates in HSC although it has recently been suggested that stellate cells become activated Ca²⁺ signalling pathways take on a more important role.³⁹ Regardless of which pathway predominates, there is already sufficient evidence to demonstrate that both make important contributions and, as a result, this review will summarise data from HSC, muscle, and non-muscle cell types. To date, a number of agents which elicit either HSC contraction or relaxation have been identified (table 1). In fig 2, intracellular pathways of contraction and relaxation are summarised.

Mechanisms of contraction

Phosphoinositidase C

Activation of phosphoinositidase C, often referred to as phospholipase C (PLC), results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) and the production of inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃

elevates [Ca²⁺]_i by releasing Ca²⁺ from intracellular stores and DAG activates PKC. Thus the hydrolysis of PIP₂ generates two important second messengers: calcium and PKC.⁴⁰

(i) Calcium is elevated either through hydrolysis of inositol lipids (as described above) or by entry through receptor/voltage gated channels in the plasma membrane. Contractile agents have been demonstrated to elicit inositol lipid hydrolysis in HSC suggesting a role for this signalling pathway in HSC contraction.^{7,41} In addition, a recent study has demonstrated that activation of HSC is associated with both upregulation of L type voltage operated Ca²⁺ channels that mediate Ca²⁺ influx and KCl induced contraction.⁴² However, as HSC have not been shown to be excitable, it will be important to establish the physiological relevance of these channels. It has been assumed that Ca²⁺ signalling pathways play an important role in mediating the contractile force generated by HSC in a similar manner to that shown in smooth muscle.⁴³ Elevation of [Ca²⁺]_i elicits Ca²⁺/calmodulin dependent activation of myosin light chain (MLC) kinase (see below) and dissociation of caldesmon from the actin-caldesmon-tropomyosin-myosin fibrillar domain which results in cycling cross bridge formation between actin and phosphorylated light chains of myosin. It has been demonstrated that reductions in HSC area, a marker of contractile force generation, correlate with the height of the [Ca²⁺]_i peak.⁴⁴ However, many measurements of contraction are made 5–6 hours after agonist addition and it is therefore important to consider mechanisms which may regulate sustained contraction. In smooth muscle, the initial and sustained phases of contraction are mediated by different cellular and molecular events. The height of the Ca²⁺ transient correlates not only with the peak rise in MLC phosphorylation (see below) but also PKC association with the plasma membrane.⁴⁵ This latter component has not been assessed in HSC.

(ii) Activation of PKC in smooth muscle by phorbol ester results in a slowly developing and sustained contractile response.⁴⁶ The time of initiation is shortened, rate of rise in tension is increased, and the magnitude of response is enhanced by agents (for example, ionomycin) that increase Ca²⁺ influx. Moreover, in chemically skinned vascular smooth muscle, phorbol ester induces a slowly developing contractile response at constant Ca²⁺ without any change in MLC phosphorylation.⁴⁷ The production of a sustained response by PKC appears to involve phosphorylation of intermediate filaments (for example, desmin, synemin), caldesmon, and a small number of unidentified low molecular weight proteins.⁴⁵ The role of PKC in HSC contraction has received little attention although its involvement is supported by the demonstration that inhibition of PKC prevents the response to ET-1.⁴⁸

Phospholipase D (PLD)

Evidence has recently emerged in support of the involvement of a further signalling pathway downstream of PKC. Stimulation of PKC results in activation of PLD and production of the second messenger phosphatidic acid (PA).⁴⁹ Addition of exogenous PLD or PA to quiescent fibroblasts elicits the formation of actin stress fibres and PLD mediates PA induced stress fibre formation in these cells.^{50,51} Moreover, PLD localises with F-actin and three proteins, vinculin, talin, and α -actinin, found on the cytoplasmic face of focal adhesions suggesting a role in the assembly/regulation of these structures.⁵²

"The data strongly suggest that investigations into the involvement of PKC/PLD in HSC contraction are likely to be fruitful areas of research for the future"

Overexpression of PLD results in cytoskeletal reorganisation consistent with a regulatory role in contraction.⁵³ Interestingly, PLD is regulated by both of the major pathways

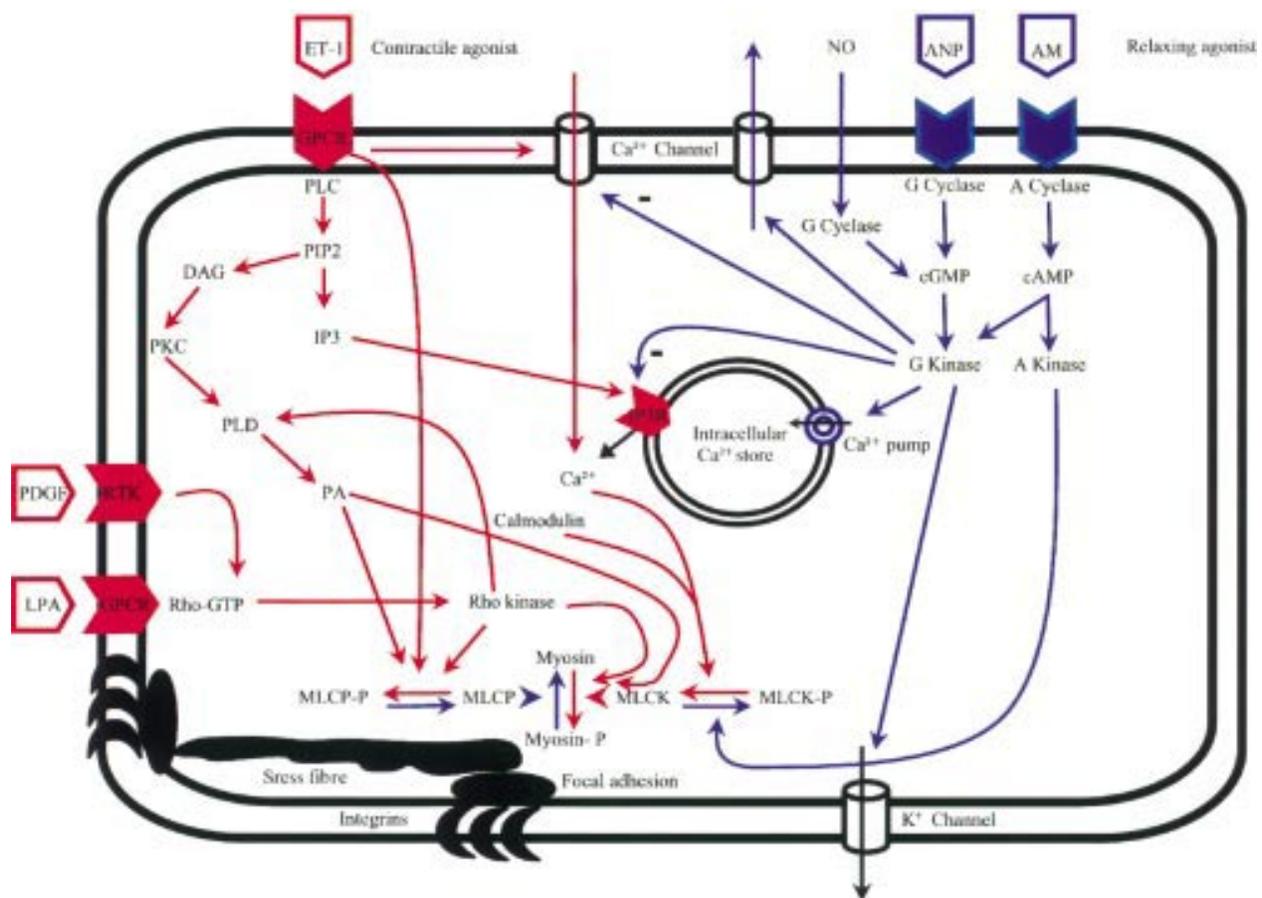


Figure 2 Schematic representation of intracellular contractile and relaxation mechanisms in hepatic stellate cells. It should be noted that most of the pathways have been described in smooth muscle cells but not all mechanisms have been proved to exist in stellate cells. Pathways for contraction are marked in red whereas blue represents relaxation pathways. AM, adrenomedullin; ANP, atrial natriuretic peptide; DAG, diacylglycerol; ET-1, endothelin 1; GPCR, G protein coupled receptor; IP₃, inositol 1,4,5-trisphosphate; LPA, lysophosphatidic acid; NO, nitric oxide; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; PIP₂, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PLD, phospholipase D; PKC, protein kinase C; RTK, receptor tyrosine kinase.

implicated in the control of contraction: a Ca²⁺ dependent kinase (that is, PKC) and rho (see below). Taken together, the data strongly suggest that investigations into the involvement of PKC/PLD in HSC contraction are likely to be fruitful areas of research for the future.

Phosphatidylinositol 3-kinase (PI(3)K)

To date, very few studies have investigated the possible involvement of PI(3)K in cell contraction. Evidence demonstrates that PI(3)K is recruited to focal adhesions (see below) where it plays a structural/signalling role. The products of PI(3)K, phosphatidylinositol (3,4) bisphosphate (PI(3,4)P₂) and phosphatidylinositol (3,4,5) trisphosphate (PI(3,4,5)P₃) have been shown to mediate actin assembly in platelets. Their role, if any, in HSC contraction is presently unknown.

Rho

Rho is a member of the Ras superfamily and recent data demonstrate that activation of rho dependent responses is primarily mediated through G proteins of the G α 12/13 family.⁵⁴ A number of rho effectors have been identified and the two serine/threonine kinases (rho kinase/ROK α /ROCK-II, and p160ROCK/ROCK β), commonly referred to as rho kinase, are the best characterised.⁵⁴ Rho regulates cell morphology through organising the actin cytoskeleton and control of actomyosin dependent cellular processes.⁵⁵ Rho kinase has been shown to participate in the induction of stress fibre and focal adhesion formation and cell contraction.^{56, 57} Furthermore, rho

kinase also phosphorylates intermediate filaments, such as glial fibrillary acidic protein and vimentin.⁵⁸ In HSC, rho and rho kinase have been shown to enhance myosin activation suggesting a role in the generation of contractile force.⁵⁹ This possibility has been supported by the demonstration that rho signalling pathways regulate agonist (for example, ET-1, lysophosphatidic acid (LPA)) induced shrinkage of collagen gels.^{59, 60} Moreover, HSC activation was accompanied by rho induced formation of actin stress fibres and focal adhesions.⁶¹

Studies in smooth muscle have described an intriguing role for rho. Work with permeabilised blood vessels demonstrated that rho kinase mediated Ca²⁺ sensitisation and directly modulated smooth muscle contraction through MLC phosphorylation independently of the Ca²⁺-calmodulin MLC kinase pathway.⁶² Clearly, this is an area which requires further investigation in HSC.

Recruitment of signalling proteins

In fibroblasts, three of the agents shown to elicit HSC contraction (ET-1, vasopressin, and LPA) rapidly stimulate both (i) tyrosine phosphorylation of p125 focal adhesion kinase (FAK) and paxillin and (ii) their clustering at focal adhesions, in a Ca²⁺ and PKC independent manner.^{63, 64} Evidence suggests that increased tyrosine phosphorylation is a consequence of activation of a tyrosine kinase situated upstream of rho and coupled to G α 13.⁶⁵ This results in the creation of binding sites for, and clustering of, other proteins—for example, src kinase and PI(3)K—at focal adhesions for structural/signalling

functions.⁶⁶ To date, protein phosphorylation (on tyrosine, serine, or threonine residues) in relation to contraction/relaxation has received little attention in HSC.

Myosin light chain

As stated above, phosphorylation of MLC is a key pathway for actomyosin interaction. This is mediated by either Ca^{2+} /calmodulin dependent MLC kinase or rho kinase. The former directly phosphorylates MLC while the latter also acts through this mechanism and/or by regulating the activity of myosin phosphatase.^{67,68} In HSC, phosphorylation of MLC by LPA correlates with its effects on contraction. Both responses are inhibited by the rho kinase inhibitor Y-27632, implying a link between rho kinase, MLC phosphorylation, and contraction.⁵⁹

Mechanisms of relaxation

A survey of the literature shows that, in comparison with contraction, little is known of the signalling pathways which regulate HSC relaxation. In view of the proposed role of HSC contraction in portal hypertension, a detailed investigation into the mechanisms underlying HSC relaxation may ultimately have major clinical implications.

Cyclic adenosine monophosphate (cAMP)

An increase in cAMP appears to reduce HSC contraction. A cAMP dependent protein kinase has been reported to inactivate MLC kinase by phosphorylating two sites of the protein⁶⁹ and, moreover, the cell permeable cAMP analogue, dibutyryl cAMP, induces the disappearance of actin stress fibres in contracted HSC.⁴⁸

"An increase in cAMP appears to reduce HSC contraction"

At high concentration, cAMP can activate a cGMP dependent kinase (G kinase). Moreover, cAMP reduces Ca^{2+} influx, possibly by a direct effect on L type Ca^{2+} channels.⁷⁰

cGMP

Elevation of NO, causing activation of guanylate cyclase, an increase in cGMP, and activation of G kinase appears to underlie the ability of sodium nitroprusside and interleukin 1 to promote HSC relaxation.^{25,48} In smooth muscle cells, G kinase has been shown to decrease $[\text{Ca}^{2+}]_i$ by several mechanisms: (i) decreasing Ca^{2+} influx by inhibiting L type Ca^{2+} channels, (ii) attenuation of IP₃ induced Ca^{2+} release from intracellular stores, and (iii) increasing Ca^{2+} efflux by activation of Ca^{2+} ATPase.⁷⁰ Moreover, sodium nitroprusside also induces disappearance of actin stress fibres in contracted HSC and nitric oxide has been reported to disrupt focal adhesion complexes.⁷¹

MLC kinase and the formation of stress fibres/focal adhesions

Stimulation of fibroblasts with LPA increases MLC phosphorylation with a time course preceding that for the detection of tyrosine phosphorylation, stress fibres, and focal adhesions. Moreover, pharmacological inhibition of MLC kinase prevented formation of stress fibres and focal adhesions, implying that MLC phosphorylation plays an important role in the formation/stability of these structures.⁷² Thus inhibition of MLC kinase by a cAMP/cGMP dependent kinase may underlie the disappearance of stress fibres/focal adhesions in activated HSC.

It is clear that the regulation of events described in this review results from cross talk between a number of signalling pathways. This is true not only for the equilibrium between contraction/relaxation but is also likely to be the case for what appear to be the two main signalling systems (Ca^{2+} /rho) which control contraction. A detailed understanding of the interplay

between these systems represents an important step towards the ultimate goal of designing novel treatments aimed at treating portal hypertension.

VASOACTIVE SUBSTANCES INFLUENCING HSC CONTRACTILITY

The effects of vasoactive substances have been extensively studied in activated stellate cells. One of the most potent and certainly the most studied vasoconstrictor is ET-1. Endothelins are a family of three homologous oligopeptides of 21 amino acid polypeptides (ET-1, ET-2, and ET-3) which are cleavage products of larger precursor proteins, cleaved by endothelin converting enzyme.^{73,74} The peptides act through at least two G protein coupled receptors, termed type A (ET_A) and type B (ET_B), with ET_B receptor having two isoforms: ET_{B1} and ET_{B2} .^{75,76} The affinity of ET-1 for the ET_A receptor is 100-fold higher than that of ET-3, whereas the ET_B receptor has similar affinity for ET-1, ET-2, and ET-3.⁷⁶ The ET_A receptor is mainly localised on smooth muscle cells and mediates principally vasoconstriction while ET_B receptors are present on a variety of cells and have several biological effects. The ET_{B1} receptor induces endothelial cell nitric oxide synthetase (eNOS) resulting in NO release and relaxation whereas ET_{B2} receptors cause vasoconstriction.^{18,75,77} Both ET_A and ET_B receptors were present on HSC and hepatocytes while only ET_B receptors were present on sinusoidal endothelial cells and Kupffer cells.⁷⁸⁻⁸¹

"In cirrhotic patients, a direct relationship between ET-1 mRNA receptor abundance and the degree of portal hypertension has been documented"

Compared with normal livers, the number of both ET receptors was increased in cirrhotic livers.⁸² In cirrhotic patients, a direct relationship between ET-1 mRNA receptor abundance and the degree of portal hypertension has been documented.⁸³ Splanchnic ET-1 concentration was proportional to the degree of portal hypertension.⁸⁴ In addition, increased plasma levels of ET-1 in patients with cirrhosis have been documented by several investigators, and the grade of ET-1 elevation correlated with the severity of cirrhosis, presence of ascites, and presence of the hepatorenal syndrome.⁸⁴⁻⁹⁵ Very high levels of ET-1 have been measured in the splanchnic vessels but the highest levels of ET-1 have been found in hepatic veins.^{84,90,95} In patients with portal hypertension, at least part of ET-1 is produced in the gut and spleen.⁹⁵ Moreover, it has been shown that ET-1 synthesis occurred within the liver. Indeed, cultured sinusoidal endothelial cells and hepatocytes have been shown to release ET-1.^{81,96} Both ET-1 mRNA and protein were markedly elevated in cirrhotic liver tissue. Activated HSC have been shown to be a major site of ET-1 synthesis via differential regulation of endothelin converting enzyme 1 mRNA and protein.⁹⁷⁻⁹⁹ Furthermore, during activation of HSC, sensitivity to ET-1 was increased.^{27,28} Enhanced ET-1 synthesis and release, and increased sensitivity to ET-1, suggest the possibility of a paracrine or autocrine action of ET-1.⁹⁸

"Short term blockade of ET receptors by mixed ET_A/ET_B receptor antagonists decreased portal hypertension in some models of experimental cirrhosis"

In the early phase of activation of HSC, ET_A receptors were predominant but with increasing activation ET_B receptors became increasingly more abundant.⁹⁷ This shift in receptor densities might be important in view of the different effects of ET-1 during the process of activation of HSC. Stimulation of ET_A receptors caused contraction and proliferation of HSC whereas ET_B receptor stimulation resulted in antiproliferative effects and relaxation.^{97,100,101} In cultured HSC, ET-1 induced a

dose dependent increase in $[Ca^{2+}]_i$ both by release of Ca^{2+} from intracellular stores and by stimulating Ca^{2+} influx coupled with contraction of the cells.⁵ ET-1 was shown to elicit sustained vasoconstriction of the hepatic vasculature.¹⁰² Short term blockade of ET receptors by mixed ET_A/ET_B receptor antagonists decreased portal hypertension in some models of experimental cirrhosis.^{29 103-105} However, these results could not be confirmed following chronic blockade of ET receptors.¹⁰⁶ Indeed, in cirrhotic rats treated with an ET receptor antagonist no differences in portal pressure were found but higher hepatic hydroxyproline content and procollagen type I mRNA expression were demonstrated.

NO is an omnipresent messenger molecule involved in various cellular processes, including neurotransmission, inflammation, and regulation of vascular tone. It is a potent vasodilator, acting in a paracrine fashion by directly stimulating soluble guanylate cyclase, resulting in increased levels of cGMP and consequently decreased $[Ca^{2+}]_i$ and vasorelaxation. Although NO has a short half life, it has prolonged effects by metabolites such as peroxynitrate. Nitric oxide is produced from L-arginine by three isoforms of nitric oxide synthetase (NOS). A wide variety of cells, including vascular smooth muscle cells, hepatocytes, and HSC, express the inducible form of NOS (iNOS) whereas endothelial cells (eNOS) and neuronal cells (nNOS) express constitutive NOS forms.^{107 108} Several growth factors (for example, transforming growth factor β , vascular endothelial growth factor), hypoxia, and shear stress increase eNOS mRNA while tumour necrosis factor α decreases its expression. The constitutive isoforms respond to changes in $[Ca^{2+}]_i$ and produce small amounts of NO. The iNOS isoform is modulated by a variety of stimuli, including cytokines and lipopolysaccharide. The enzyme binds calmodulin at almost all $[Ca^{2+}]_i$ concentrations and NO production is high.^{107 109} In cirrhosis, systemic and splanchnic vascular NO production is increased resulting in the development of a hyperdynamic syndrome. It appears that eNOS is responsible for the enhanced NO synthesis but the cause is not entirely clear.¹⁰⁸ In contrast with the high NO production in the systemic and splanchnic vascular system, NO production within the cirrhotic liver is deficient. It has been demonstrated that NO modulates hepatic vascular tone in normal liver, and that sinusoidal endothelial cells express both in vitro and in vivo eNOS and produce NO.^{110 111} Not only are sinusoidal endothelial cells responsible for basal NO production but also for increased release of NO in response to flow and shear stress.¹¹¹ Exogenous NO abolished the vasoconstrictive effects of ET-1 in the in vitro perfused rat liver.^{14 112} In the cirrhotic liver however, endothelial dysfunction with decreased NO synthesis by sinusoidal endothelial cells caused by decreased enzymatic function of eNOS has been reported.¹¹³⁻¹¹⁵ NO was shown to modulate the contractile effect of ET-1 on cultured HSC.^{48 116} Interleukin 1β , tumour necrosis factor α , or lipopolysaccharides in combination with interferon γ led to increased iNOS mRNA levels in HSC and increased nitrite levels, possibly by an autocrine mechanism.¹¹⁶ However, iNOS mRNA and nitrite were not found in HSC cultured from CCl_4 treated rat livers.¹¹⁷ Apart from vasoactive effects, deficient NO in cirrhosis has some other effects: (i) increased thrombogenesis, (ii) enhanced synthesis and deposition of collagen in the space of Disse, (iii) decreased suppression of specific adhesion molecules, and (iv) decreased protection against free oxygen radicals.¹⁰⁸

Although endothelins and NO are probably the most important and certainly the most studied vasoactive substances, several other substances have been investigated. In denervated, isolated, perfused rat liver, atrial natriuretic peptide (ANP) antagonised the increased intrahepatic vascular resistance elicited by phenylephrine.¹¹⁸ Cultured human HSC have receptors for ANP and activation of these receptors reduced the effects of ET-1 on $[Ca^{2+}]_i$ and cell contraction.^{41 119} The relaxing effects were shown to be mediated by C type ANP

inducing an increase in cGMP and by blockade of store operated calcium channels.⁴¹ Adrenomedullin is a potent vasodilator of which circulating plasma levels have been shown to be elevated in cirrhosis, as a function of haemodynamic changes.^{120 121} It has been demonstrated that human HSC have functional receptors for adrenomedullin, stimulation of which blunted the contractile effect of ET-1.¹²² Adrenomedullin exerts its relaxing effects in HSC through cAMP cascades.¹²³ Interestingly, both cytokines and shear stress augmented expression of C type ANP and adrenomedullin.¹²⁴ Somatostatin is used to treat acute variceal bleeding. Somatostatin inhibits the secretion of glucagon, a well known vasodilating agent. Moreover, in the presence of vasoconstrictors involving activation of PKC, octreotide had a vasoconstrictive effect on vascular smooth muscle of the superior mesenteric artery.¹²⁵ We have shown that, in addition to the effects on portal blood flow, activation of the somatostatin receptor subtype 1 caused partial inhibition of ET-1 induced contraction of stellate cells.¹²⁶

Several other substances have been investigated in vitro but the in vivo significance on portal pressure remains unclear. Vascular endothelial growth factor inhibited contraction of cultured HSC by attenuating α smooth muscle actin expression.¹²⁷ Agents increasing intracellular cAMP (for example, adrenomedullin, Iloprost, prostaglandin E_2) and cGMP (for example, lipopolysaccharide, interferon γ , interleukin β , ANP, NO) induce relaxation of stellate cells.^{25 41 48 122 128 129}

“Although different effects on portal pressure have been claimed, to date no definite role in portal hypertension has been attributed to most eicosanoids”

Some prostaglandins (for example, PGI_2 , PGE_2) induced relaxation of cultured HSC whereas others (for example, thromboxane, $PGF_{2\alpha}$) induced contraction.¹²⁸ Although different effects on portal pressure have been claimed, to date no definite role in portal hypertension has been attributed to most eicosanoids. Administration of leukotriene D_4 , a 5-lipoxygenase derived eicosanoid, resulted in marked HSC contraction coupled with increased $[Ca^{2+}]_i$. Moreover, leukotriene D_4 administration significantly increased portal pressure in rats.¹³⁰

Antidiuretic hormone or vasopressin, a potent vasoconstrictor acting through V_1 receptors on vascular smooth muscle cells, was successfully used to treat acute variceal bleeding. Vasopressin also elicited contraction of cultured human HSC through V_1 receptors. Stimulation of V_1 receptors is coupled with increased $[Ca^{2+}]_i$ caused by release of Ca^{2+} from intracellular stores.¹³¹ Similar to vasopressin, thrombin induced dose dependent contraction of activated human HSC.^{5 41} Thrombin induced contraction is coupled with a rise in $[Ca^{2+}]_i$ via release of Ca^{2+} from intracellular stores and via store operated⁴¹ or voltage operated Ca channels.⁵ It has been known for many years that the renin-angiotensin system plays a role in increased intrahepatic vascular resistance and that the activity of the renin-angiotensin system correlates with the degree of portal hypertension.¹³² Angiotensin II (ANGII) is an octapeptide primarily acting through type 1 angiotensin receptor (AT1).¹³³ Infusion of ANGII resulted in increased intrahepatic vascular resistance and portal hypertension in experimental cirrhosis. In activated, but not in quiescent, human HSC, ANGII induced an AT1 mediated dose dependent increase in $[Ca^{2+}]_i$ accompanied by proliferation and cell contraction.^{5 134} In rat HSC however, ANGII did not induce contraction of collagen lattices.²⁸ Either rat HSC do not bear AT1 or the method used to measure contraction was not sufficiently sensitive. Substance P caused a dose dependent contraction of rat HSC cultured for 24 hours.²⁶ Substance P was increased in decompensated cirrhotic rats and patients, although no direct relation with portal pressure was

found.¹³⁵⁻¹³⁶ Stellate cells have nucleotide receptors which are triggered by adenosine, resulting in receptor mediated contraction of the cells.¹³⁷ LPA, a product of phospholipid metabolism released by activated platelets, was shown to induce HSC contraction by a mechanism involving rho kinase.⁵⁹

Although activated HSC contract in response to vasoactive substances, both in vivo and in vitro, there has been some debate as to whether stellate cell contraction is forceful enough to produce sinusoidal constriction. This issue was resolved in a recent study in which stellate cell contraction was directly quantitated.¹³⁸ It was demonstrated that the average force generated by ET-1 induced contraction of a single HSC was ~ 0.69 dyn or 14000 dyn/cm², which is greater than the sinusoidal pressure in cirrhotic rats, indicating that HSC can constrict sinusoids and thus contribute to increased sinusoidal resistance.

PHARMACOLOGICAL TREATMENT OPTIONS DIRECTED TOWARDS HSC

Fibrosis remains the principal cause of increased vascular resistance in liver disease. Because HSC play a key role in the development of liver fibrosis, much effort has been made in the development of drugs acting on HSC in order to prevent or revert the process of fibrosis. Potential therapies are currently under investigation.¹³⁹

“Most drugs are directed towards lowering portal blood flow but new drugs acting on HSC contractility are being investigated”

Although clinical experimental studies indicate that hepatic fibrosis is partially reversible, complete resolution of cirrhosis with re-establishment of normal liver histology will probably not be possible. Hence portal hypertension and its complications will continue to be a major challenge for clinicians. Therefore, effective antiportal hypertensive drugs are needed. Currently, most drugs are directed towards lowering portal blood flow but new drugs acting on HSC contractility are being investigated. Ideally, a drug should be antifibrotic, induce stellate cell relaxation, and decrease portal blood flow. Some potential candidates will be discussed briefly.

In view of the beneficial effects of endothelin via activation of ET_B receptors and the negative effects of ET_A receptor stimulation (contraction and proliferation of HSC), selective blockade of ET_A receptors would be a good approach to treating portal hypertension. Oral administration of a selective ET_A receptor antagonist ameliorated portal hypertension in portal vein ligated rats¹⁴⁰ and reduced fibrosis in a bile duct ligation model.¹⁴¹ So far no solid clinical data are available in cirrhotic patients. Although ET_B receptor agonists and endothelin converting enzyme inhibitors are theoretically very interesting molecules, up to now no in vivo data are available.

Apart from the relaxing effects of NO on HSC, antiproliferative effects of NO have been demonstrated in vitro.¹⁴² Although NO causes relaxation of HSC, it also increases splanchnic blood flow, and systemic non-specific NO donors will probably not be clinically useful in treating portal hypertension. Because of local intrahepatic NO deficiency, another approach has been explored. Transduction of livers with recombinant adenoviruses carrying the nNOS gene significantly reduced intrahepatic resistance and portal pressure in two different in vivo models of cirrhosis and portal hypertension.¹⁴³

Losartan, a specific ATI receptor antagonist, completely blocked the effects of ANGII on HSC contraction and proliferation and induced a significant decrease in portal hypertension in cirrhotic patients.¹³⁴⁻¹⁴⁴ Long term effects and side effects have to be examined.

However, long term losartan administration did not significantly reduce hepatic vein pressure gradient in cirrhotic

patients. Moreover, it caused hypotension and reduced glomerular filtration rate in these patients. Therefore, losartan does not seem to be a good treatment option.¹⁴⁵

Inhibition of rho signalling by a specific inhibitor of rho kinase (Y-27632) decreased HSC contraction, proliferation, migration, and activation associated morphological alterations.^{60-61, 146-147} It also reduced ET-1 induced portal vein constriction in an ex vivo liver perfusion model but no in vivo experiments have been published so far.⁶⁰

Somatostatin is a well established drug to treat acute variceal haemorrhage. Apart from vasoconstrictive effects on mesenteric arteries¹²⁵ and relaxing effect on rat stellate cells,¹²⁶ antifibrotic effects have been attributed to somatostatin analogues in in vivo animal models.¹⁴⁸ We have demonstrated decreased production of collagen I and III by cultured HSC in the presence of somatostatin, suggesting that somatostatin also has a direct antifibrotic effect.¹⁴⁹

CONCLUSION

Hepatic stellate cells are key players in the pathogenesis of fibrosis and portal hypertension. Hence HSC have become therapeutic targets to treat these complications of chronic liver disease. HSC probably also play a role in the normal liver microcirculation. The contractile apparatus of HSC and the signalling pathways that control contraction or relaxation require further in depth studies. Antifibrotic and vasomotor effects of multiple agents have been exhaustively investigated, predominantly in vitro and in experimental animal models. These studies have led to the identification of drugs causing inhibition of activation and relaxation of HSC, thereby decreasing fibrosis and portal pressure. Based on this experimental work, clinical studies in patients will have to be undertaken.

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