Review article

The profibrotic role of endothelin-1: Is the door still open for the treatment of fibrotic diseases?

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ABSTRACT

The endothelin (ET) system consists of two G-protein-coupled receptors (ET_A and ET_B), three peptide ligands (ET-1, ET-2 and ET-3), and two activating peptidases (endothelin-converting enzyme-, ECE-1 and ECE-2). While initially described as a vasoregulatory factor, shown to influence several cardiovascular diseases, from hypertension to heart failure, ET-1, the predominant form in most cells and tissues, has expanded its pathophysiological relevance by recent evidences implicating this factor in the regulation of fibrosis. In this article, we review the current knowledge of the role of ET-1 in the development of fibrosis, with particular focus on the regulation of its biosynthesis and the molecular mechanisms involved in its profibrotic actions. We summarize also the contribution of ET-1 to fibrotic disorders in several organs and tissues. The development and availability of specific ET receptor antagonists have greatly stimulated a number of clinical trials in these pathologies that unfortunately have so far given negative or inconclusive results. This review finally discusses the circumstances underlying these disappointing results, as well as provides basic and clinical researchers with arguments to keep exploring the complex physiology of ET-1 and its therapeutic potential in the process of fibrosis.

Contents

Introduction

Signal transduction processes are essential in both single-celled and multicellular organisms for sensing and coordinating responses to changes in their environment. One of the largest and most diverse cellular systems serving to communicate with the external milieu is the superfamily of G-protein coupled receptors (GPCR). GPCR, which are encoded by more than 800 genes in the human genome constitute a family of membrane proteins capable of detecting a wide spectrum of extracellular signals, including photons, ions, small organic molecules and entire proteins or peptides (for a recent review, see Katritch et al., 2013). Contemporary to the studies on the identification and characterization of GPCR by Robert J. Lefkowitz, who received together with Brian K. Kobilka the Medicine Nobel Prize 2012 for this seminal contribution, a

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group of enthusiastic cardiovascular researchers dedicated their effort, following the discovery of endothelial-derived relaxing factor by Robert F. Furchgott in 1980, on identifying new vasoactive factors with constrictor capabilities (Cerione et al., 1983; Furchgott and Zawadzki, 1980; Masaki, 2004). As a result of this search, a novel peptide named endothelin (ET) was successfully purified and characterized in 1988, and only two years later, two cDNAs encoding the receptors were cloned and assigned to the family of GPCR (Arai et al., 1990; Sakurai et al., 1990; Yanagisawa et al., 1988). In humans, it is now clear that endothelin represents the most potent and long-lasting vasoconstrictor known, 100 times more potent than noradrenaline, coincidentally the ligand of the first GPCR described by R.J. Lefkowitz (Luscher and Barton, 2000). The result of this fruitful investigation was the characterization of the endothelin system, consisting of two GPCR (ET$_A$ or EDNRA and ET$_B$ or EDNRB), three peptide ligands (ET-1, ET-2 and ET-3), and two activating peptidases (endothelin-converting enzyme-, ECE-1 and ECE-2) (Miyauchi and Masaki, 1999). In relation with its first described vasoregulatory role, ET system has been shown to influence several cardiovascular diseases, including chronic and ischemic heart failure, systemic and pulmonary hypertension, atherosclerosis, chronic renal failure and cerebrovascular disease, and beyond the cardiovascular system, the early development of the neural crest and, thus, the formation of organs (Kedzierski and Yanagisawa, 2001; Rodriguez-Pascual et al., 2011).

One step forward in the investigation of the pathophysiological role of the ET system was the realization that significantly high levels of ET-1, the predominant isoform in most cells and tissues, are persistently observed in a number of fibrotic diseases (Kawaguchi et al., 1994; Shi-Wen et al., 2006). This review summarizes the current knowledge of the role of ET-1 in the development of fibrosis, with particular emphasis in the regulation of the biosynthesis of ET-1 in the fibrotic milieu, the molecular mechanisms described to be involved in its profibrotic actions, and the contribution of ET-1 to fibrotic disorders in several organs and tissues.

General concepts on fibrosis: where and how the endothelin system acts

Over recent years, our understanding of the functions of the extracellular matrix (ECM) has evolved from the traditional concept of a static "glue" holding cells into tissues to the more sophisticated one of a dynamic biomaterial that provides strength and elasticity, as well as points of interactions with cell surface receptors, and availability of growth factors. Proper formation and organization of the ECM is essential for cell and tissue homeostasis. Matrix-related diseases arise from both defects in the properties of ECM components, as occurs in congenital diseases such as Marfan syndrome and Ehlers–Danlos syndrome, as well as in conditions characterized with an excess of production and deposition of ECM components. This excess, generally termed as fibrosis, is a pathophysiological circumstance that is common to a number of chronic diseases including idiopathic pulmonary fibrosis, liver cirrhosis, systemic sclerosis and nephrosclerosis as well as several cardiac diseases (Bateman et al., 2009; Varga et al., 2005).

The fibrotic process is the result of an aberrant response to injury. The immediate response to an insult is the activation of a complex system of cellular responses whose ultimate goal is the fast and efficient repair of the tissue. The physiological response of tissues to damage is very complex and displays context-specific features. However, there are a number of events that are common to different tissues, and to a wide variety of causative agents (Wynn, 2007). Essentially, the process progresses through 3 well-established steps: 1) an initial phase of injury, 2) a second phase characterized by inflammation, and 3) a remodeling step, which includes synthesis and deposition of ECM components by myofibroblasts. These cells share many characteristics with smooth muscle cells, including their morphology and contractile capacity, are located at sites of active fibrosis and are responsible for the synthesis and deposition of ECM

proteins (Gabbiani, 2003; Hinz, 2010). Myofibroblasts have been shown to be able to derive from several cell types including resident fibroblasts, circulating fibrocytes, epithelial/endothelial cells undergoing epithelial/endothelial-to-mesenchymal transition, EMT/EndoMT, vascular pericytes or hepatic stellate cells (Wynn, 2008). ET-1 has been able to promote the induction of the myofibroblast phenotype in these cell types (Fig. 1). For example, resident fibroblasts have been shown to transdifferentiate to myofibroblasts in response to ET-1 in several tissues, including the lung, heart and skin (Lagares et al., 2012a, 2012b, 2010; Nishida et al., 2007). Additionally, ET-1 has been also described to promote EMT/EndoMT in epithelial/endothelial cells as a source of myofibroblasts in the fibrotic mileau (Jain et al., 2007; Piera-Velazquez et al., 2011; Sun et al., 1997). Bone marrow derived monocytes have also been reported to undergo myofibroblast transition in response to ET-1 (Binai et al., 2012). In the context of liver fibrosis, hepatic stellate cells have been shown to transform into fibrotic fibroblasts by the action of ET-1 (He et al., 2013; Zhan and Rockey, 2011). Finally, vascular pericytes, contractile cells wrapping around endothelial cells within capillaries in the microvasculature, have been recently proposed to play an important role in tissue fibrosis giving rise to myofibroblasts in response to several factors, including ET-1 (Fligny and Duffield, 2013; Simonson and Ismail-Beigi, 2011).

The acquisition of the myofibroblast phenotype endows the fibrotic cell with several capabilities, including enhanced contractility, migration and synthesis and deposition of ECM components. To this respect, early work done before the extent of role of ET-1 in the onset of fibrosis was even recognized; described the capacity of ET-1 to enhance the expression of collagen isoforms in several cells and tissues, including cardiac fibroblasts and smooth muscle cells, among others (Guarda et al., 1993; Mansoor et al., 1995; Rizvi et al., 1996). These observations have been later corroborated in studies performed in heart, skin, lung, liver or even in the eye within the lamina cribrosa (Ammarguellat et al., 2001; Guo et al., 2004; Hafizi et al., 2004; Horstmeyer et al., 2005; Nishida et al., 2007; Rao et al., 2008; Shi-wen et al., 2007; Simonson and Ismail-Beigi, 2011; Xu et al., 1998).

In a physiological wound healing response, the cellular and acellular components of the scar are eliminated and the fibrotic deposition becomes replaced by regenerated tissue. Myofibroblast elimination by apoptosis seems to play a key role in this resolution phase, and failure in this process seems to contribute to the development of fibrosis (Kis et al., 2011). To this respect, ET-1 has been described to promote myofibroblast resistance to apoptosis and therefore contribute to the persistence of the fibrotic response (Horowitz et al., 2012).

Regulation of the expression of ET-1 in fibrosis

Having described that ET-1 is able to promote the induction of the myofibroblast differentiation, a process associated to enhanced synthesis and deposition of ECM components, it is pertinent to discuss the cells contributing to its biosynthesis and how it is regulated. By the action of a number of factors discussed below, different cell types involved in the fibrotic process, including lung, skin or cardiac fibroblasts or hepatic stellate cells, among others, have been described to generate significant amounts of bioactive ET-1 (Abraham et al., 1997; Katwa, 2003; Rockey et al., 1998; Shi-Wen et al., 2004; Shi-Wen et al., 2001). Nevertheless, most of the knowledge we have today about the mechanisms controlling the expression of ET-1 comes from research studies performed with endothelial cells, its most important biological source (Rodriguez-Pascual et al., 2011). Numerous experimental evidences implicate mRNA transcription as a major control of ET-1 biosynthesis. Several transcriptional factors including activator protein-1 (AP-1), GATA, Smad or hypoxia-inducible factor-1 (HIF-1), have been described to interact with specific genomic regions within the ET-1 gene promoter, thereby acting as mediators for the action of different biological and pharmacological factors (Inoue et al., 1989; Rodriguez-Pascual et al., 2011). Transforming growth factor-β (TGF-β), thrombin, bradykinin, angiotensin II, interferon-γ (IFN-γ), the glucocorticoids or the hypoxia upregulates ET-1 mRNA
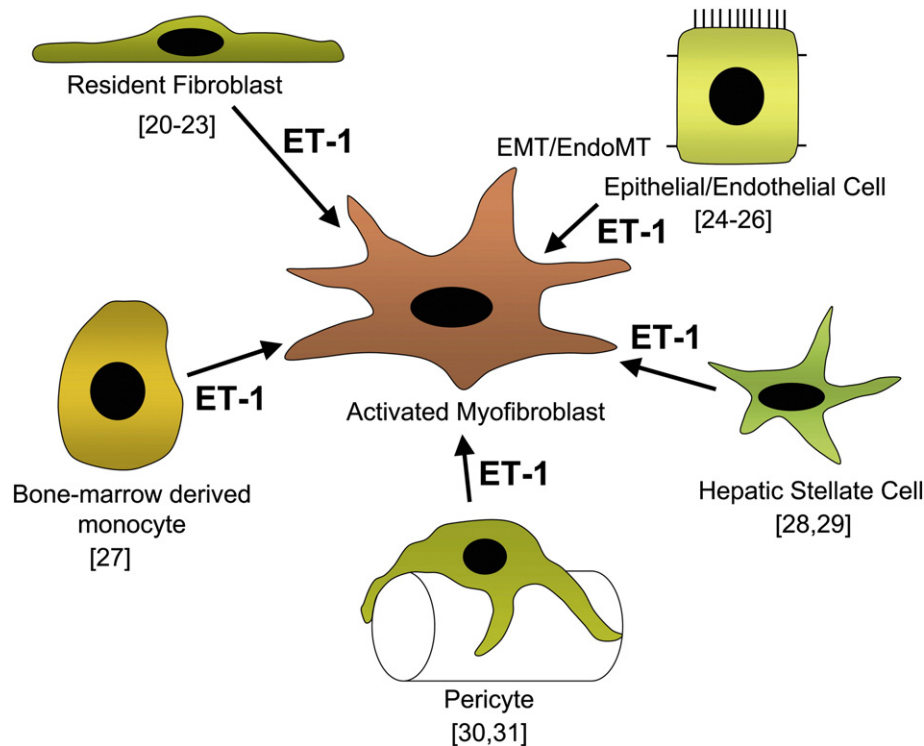
Fig. 1. Main pathways for ET-1-induced myofibroblast differentiation. Based on a number of reports (references included), ET-1 has been shown to promote the induction of the myofibroblast phenotype from resident fibroblasts, from epithelial/endothelial cells via EMT/EndoMT, from hepatic stellate cells, from circulating bone marrow-derived monocytes (fibrocytes), and from vascular pericytes.

levels; whereas vasodilator factors such as natriuretic peptides or nitric oxide, or pharmacological agents such as statins downregulate them (Miyauchi and Masaki, 1999; Stow et al., 2010). Some of these modulators are particularly interesting in the context of the fibrotic process. Thus, angiotensin II and the hypoxia, two conditions described to promote the accumulation of ECM components in several cells and tissues, have been shown to increase ET-1 expression through activation of AP-1 and HIF-1, respectively (Ahmedat et al., 2013; Hieda and Gomez-Sanchez, 1990; Hu et al., 1998; Ikeda et al., 2000; Kourembanas et al., 1991). TGF-β, which is considered a *princeps* mediator in the development of fibrosis in a wide variety of tissues, has been also reported to be one of the most potent regulators of ET-1 levels in endothelial and non-endothelial cells, this action occurring through activation of AP-1 and Smad transcription factors (Biernacka et al., 2011; Kurihara et al., 1989; Rodriguez-Pascual et al., 2003; Ruiz-Ortega et al., 2007). In fact, TGF-β-mediated ET-1 release has been associated to the fibrotic response observed in scleroderma (SSc) fibroblasts, and in the context of skin and lung fibrosis (Ahmedat et al., 2013; Kawaguchi et al., 1994; Lagares et al., 2010; Shi-Wen et al., 2006). Additional factors or signaling pathways have been also reported to modulate ET-1 expression in fibrosis. Thus, tumor necrosis factor-α (TNF-α) has been shown to increase ET-1 levels in hepatic stellate cells during hepatic wound healing (Zhan and Rockey, 2011). Endoplasmic reticulum (ER) stress and activation of the ATF4 transcription factor have been reported to promote significant increases in ET-1 expression in vascular endothelial cells from HLA-B35-expressing SSc patients (Lenna et al., 2013). Common to these findings is the activation of the AP-1 member c-Jun and further enhancement of ET-1 transcription. In fact, as extensively reported in endothelial cells, AP-1 transcription factor plays a central role in the control of constitutive and regulated transcription of the ET-1 gene, coordinating transcriptional responses to GATA-2, HIF-1 and Smad transcription factors (Kawana et al., 1995; Rodriguez-Pascual et al., 2004; Yamashita et al., 2001).

ET-1 expression is also subject of post-transcriptional regulation at the level of mRNA stability. ET-1 mRNA has been shown to be highly unstable (estimated half-life of 15 min), and specific adenine and uridine-rich elements (ARE) in the 3′-untranslated region (3′-UTR) seem to be responsible for transcript lability (Reimunde et al., 2005). Within this context, several recent reports have described regulatory effects of particular microRNA (miRNA) on ET-1 expression. Thus, miR-155 and miR-199 have been shown to downregulate ET-1 levels in liver sinusoidal endothelial cells, and miR-1 was reported to control ET-1 expression in hepatoma cells (Li et al., 2012; Yeligar et al., 2009). Whether these miRNA-mediated actions on ET-1 occur in a fibrotic milieu and are therefore of pathophysiological relevance, and also, whether a unifying mechanism exists to integrate ARE- and miRNA-mediated effects for the control of ET-1 expression, are not yet fully elucidated.

Bioactive ET-1 peptide is also regulated at the level of processing by endothelin-converting enzymes (ECE). In fact, elevated levels of ECE-1, and subsequently of ET-1, have been observed in idiopathic pulmonary fibrosis (IPF) patients, and mouse models displaying null or reduced activity or ECE-1 are resistant to develop fibrosis in the lung or heart (Hartopo et al., 2013; Kalk et al., 2011; Saleh et al., 1997).

Molecular and cellular mechanisms regulating ET-1 induced fibrosis

Biological actions of ET-1 involve necessarily the activation of specific ET_A and/or ET_B receptors. Formally speaking these receptors belong to the rhodopsin A group of GPCR, subfamily A7, and are coupled to G_q, G_{11}, G_s and G_{i2}, suggesting that endothelin receptors may simultaneously stimulate multiple effectors via several types of G protein, including phospholipase A2, C and D, as well as membrane and cytosolic protein kinases. This complexity together with the fact that most cells can coexpress both receptors, make rather difficult to predict the mechanism/s involved in the specific actions of ET-1 on a particular cell type. Nevertheless, recent experimental evidences indicate the involvement of particular cell signaling components in ET-1-induced fibrosis (Fig. 2). Thus, mitogen-activated protein kinases (MAPK) JNK and ERK, known to activate AP-1 transcription factor, have been reported to mediate the induction by ET-1 of a matrix-associated genetic program characterized by the expression of collagen isoforms, as well as of contractile proteins involved in enhanced

myofibroblast contraction and migration (Ahmedat et al., 2013; Lagares et al., 2012a, 2010; Shi-wen et al., 2007, 2006; Xu et al., 2004). Upstream MAPK, signaling components of the cell adhesion machinery, focal adhesion kinase (FAK) and integrin-linked kinase (ILK), have been described to participate in ET-1-mediated profibrotic actions (Horowitz et al., 2012; Kennedy et al., 2008; Lagares et al., 2012a, 2012b; Shafiei and Rockey, 2012). Interestingly, both proteins are activated by integrin-mediated signals from the ECM, indicating that coordinated signals coming from soluble factors and also from the environment cooperate to regulate fibrosis. A third set of signaling components is implicated in ET-1-induced tissue fibrosis. The activation of rac/Rho and phosphatidylinositide 3-kinase(PI3K)/Akt has been also shown to participate in the profibrotic actions of ET-1 (Horowitz et al., 2012; Rodriguez-Vita et al., 2005; Shafiei and Rockey, 2012; Shi-Wen et al., 2004). To add an extra level of complexity, ET-1 has been also reported to upregulate the expression of other profibrotic factors, such as connective tissue growth factor (CCN2/CTGF) or even TGF-β, actually making the fibrotic response the result of a network promoting tissue repair and fibrogenesis (Alvarez et al., 2011; Rodriguez-Vita et al., 2005; Shi-wen et al., 2007).

In addition to characterize the molecular pathways through which ET receptors drive ET-activated signals, the subtype of receptor, either ET_A or ET_B, should be taken into consideration, particularly at those scenarios where a clinical translation is pursued, and therefore, the use of a specific antagonist matters. To this respect, there exists a general consensus to favor the use of dual ET_A/ET_B blockers, rather than specific ones, biased by the fact that the only antagonist clinically approved so far belongs to the dual category, together with observations describing changes in ET_A/ET_B ratio in pathological contexts and the existence of ET_A/ET_B heterodimers (Gregan et al., 2004; Möller et al., 1999). Nevertheless, the potential benefit of selective versus dual antagonism should be experimental and clinically demonstrated in every particular model or disease.

Contribution of ET-1 to fibrosis and fibrotic disorders

As mentioned above, ET-1 has been associated to the development of fibrosis in different cell and tissue contexts. Here we summarize the current knowledge of the profibrotic action of ET-1 as classified by organ or tissue, together with the most significant clinical trials performed to test the therapeutic potential of targeting the ET system in pathologies affecting these tissues, as viewed from a fibrotic perspective.

Lung

Several lines of evidence implicate ET-1 as a mediator in the development of excessive scarring and fibrosis in the lung. Thus, plasma ET-1 levels have been found to be elevated in patients with IPF, and bronchoalveolar lavage fluids from SSc and IPF patients have been shown to present also increased ET-1 amounts (Abraham et al., 1997; Odoux et al., 1997; Uguccioni et al., 1995). Additionally, elevated expression levels of ET-1 and ET receptors have been reported in lung biopsy samples from SSc patients compared with tissues from control donors (Abraham et al., 1997). In fact, ET-1 was reported to play a role in this pathological context as fibroblasts isolated from lungs of SSc patients showed enhanced ET-1 expression and the expression of myofibroblast markers or profibrotic factors could be reduced by antagonizing ET-1 signaling (Shi-Wen et al., 2004). Reinforcing this hypothesis, data from bleomycin-induced lung fibrosis models in mice or rats have shown increased ET-1 immunoreactivity in airway epithelium and infiltrating macrophages, as well as a significant reduction in the fibrotic score in animals pretreated with endothelin receptor antagonists, and gene transfer of ET-1 in murine lungs induced a profibrotic response (Lagares et al., 2012b; Park et al., 1997). Based on this knowledge, ET-1 signaling emerged as a potential target for pharmacological intervention in the treatment of fibrosis in IPF and SSc. The first
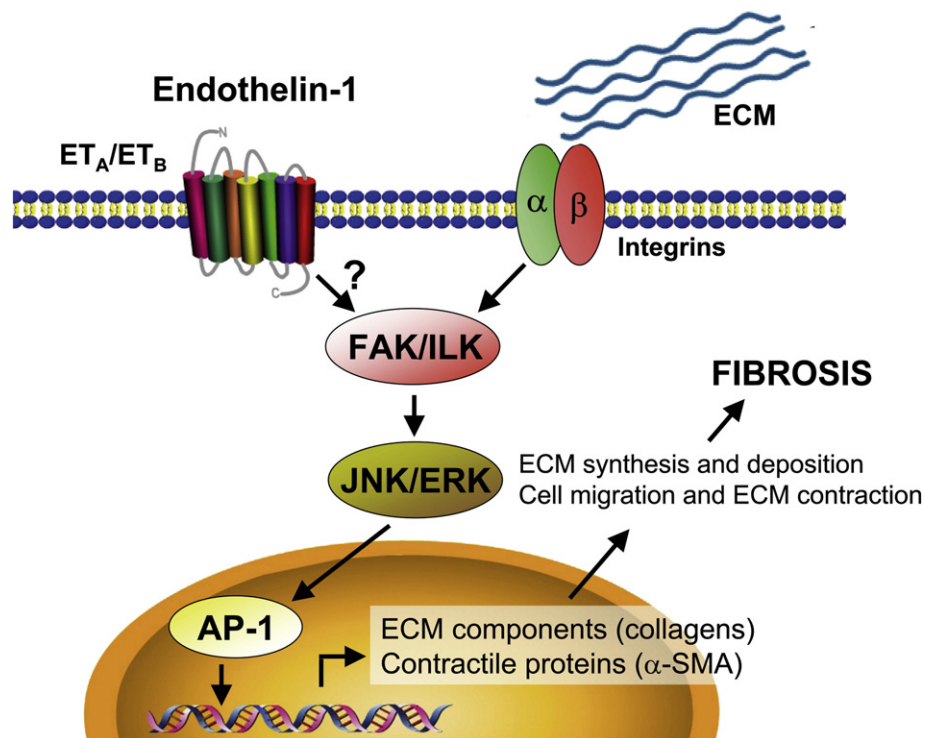
Fig. 2. Schematic diagram showing the signaling mechanisms contributing to the profibrotic actions of ET-1 as described in lung or skin fibrosis. ET-1, by acting on ET_A and ET_B receptors, has been shown to activate FAK and ILK, cell adhesion protein kinases known to receive influences from the extracellular matrix (ECM) via integrins. Downstream activation of MAP kinases, JNK and ERK, and subsequent AP-1 transcription factor are responsible for the induction of a profibrotic genetic program with expression of matrix components, for instance, collagen isoforms, as well as of contractile proteins, such as α-smooth muscle actin (α-SMA). This program, as a whole, induces the net deposition of ECM components and greatly enhances cell migration and ECM contraction, both being features of myofibroblast differentiation and fibrosis. While the molecular link between ET receptors and FAK (or ILK) remains unknown, several reports have also implicated rac/Rho and PI3K/Akt in ET-1-induced fibrosis.

international, prospective, double-blind, clinical study was the BUILD program (BUILD-1: Bosentan Use in Interstitial Lung Disease) designed to test the efficacy of this dual ET_A/ET_B receptor blocker, currently being prescribed in pulmonary arterial hypertension, to treat IPF patients (Table 1) (King et al., 2008). Primary end-point of this study was exercise capacity, whereas secondary objectives were time to death or disease progression. Bosentan showed no superiority over placebo in the exercise test (6 minute walk distance), nor in time to death or disease progression. Nevertheless, a significant improvement in the progression of the disease was observed in a patient subgroup with surgical lung biopsy-proven IPF. This observation prompted the launching of a second clinical trial (BUILD-3) restricted to patients with a confident diagnosis of IPF as they were considered most likely to respond to treatment. However, no significant difference between treatment groups was observed in the time to disease worsening (the primary end-point) (King et al., 2011). A more recent clinical trial with macitentan, a dual blocker developed from the structure of bosentan and showing increased safety and efficacy in preclinical studies, has also yielded negative results in patients with IPF or SSc (MUSIC: Macitentan USe in an Idiopathic pulmonary fibrosis Clinical study) (Raghu et al., 2013b). Additionally, a clinical study intended to determine whether ambrisentan, a selective ET_A receptor antagonist, was able to reduce the rate of IPF progression was launched with time to disease progression as primary end-point (ARTEMIS-IPF). Ambrisentan was not only unable to halt or slow down the disease worsening, but it was also linked with an increased risk for IPF progression and respiratory hospitalizations (Raghu et al., 2013a). Therefore, while there is a strong consensus within the scientific community to accept that ET-1 plays a significant role in the development of fibrosis in experimental models and a number of evidences from studies in patients point towards this pathogenic role, clinical trials so far failed to demonstrated such a role, therefore questioning the contribution of ET-1 to human pulmonary fibrosis. In fact, the nature of the lung disease is complex with influences from genetic and environmental factors, and, apart from pirfenidone acting on TGF-β signaling, no other single factor or signaling pathway has been proved to be an efficient target for the treatment of lung fibrosis (Richeldi and du Bois, 2011).

Skin

Skin fibrosis occurs in a number of human diseases, such as keloids and most notably SSc, as a result of an exaggerated wound healing response. A number of evidences indicate that ET-1 may be a mediator in the development of skin fibrosis. The binding of ET-1 has been demonstrated by autoradiography in skin biopsies from SSc patients, suggesting an overall increase in ET receptor expression (Vancheeswaran et al., 1994). In cell culture models, skin fibroblasts have been described to synthesize significant levels of ET-1 and the activation of ET receptors induces ECM production and accumulation in a process involving the cell transformation to a myofibroblast phenotype (Abraham et al., 1997; Shi-Wen et al., 2004, 2001). In addition, ET receptor blockade was shown to prevent the fibrotic response in the classical mouse model of bleomycin-skin fibrosis (Lagares et al., 2010). To this respect, it is noteworthy to mention that ET receptor antagonists were also able to prevent TGF-β-induced myofibroblast differentiation and fibrosis, therefore indicating that reciprocal regulation of TGF-β and ET-1 occurs in wound healing and skin fibrosis (Lagares et al., 2010). Translation of these experimental evidences to the clinical setting has focused on the most evident skin manifestations of SSc disease, the digital ulcers (DU) and the Raynaud's phenomenon (RP). Case reports of patients showing improvement in their RP and DU while undergoing therapy with ET blockers for pulmonary arterial hypertension led to randomized controlled trials investigating the efficacy of these agents for the treatment of RP and DU in patients with SSc. These observations paved the initiation of open-label studies and, later on, large randomized clinical trials. RAPIDS-1 and -2 (Randomized Placebo-controlled Investigation of Digital ulcers in Scleroderma) studies were performed to test the efficacy of bosentan in DU in SSc patients. Although both studies were done with an interval of seven years, they reached the same conclusion: bosentan was efficacious in preventing the formation of new DU, but it did not reduce the numbers of pre-existing ones (Korn et al., 2004; Matucci-Cerinic et al., 2011). In the case of RP, a small-scale placebo-controlled, double-blind clinical trial evaluating bosentan for the treatment of RP has disappointingly shown no significant different with the control group (Nguyen et al., 2010). Nevertheless, in the context

Table 1
Clinical trials investigating the effects of ET receptor antagonists in fibrotic (and fibrosis-related) diseases, as classified by organ or tissue. Information about the type of receptor blocker, the primary end-point/s and results/comments are also included, as well as the trial references.

Trial	Agent (type)	Primary end-point	Outcome. Comments
Lung			
BUILD-1 (King et al., 2008)	Bosentan (dual)	Exercise capacity (6MWT)	Ineffective. Improvement in 2nd. end-point: disease progression
BUILD-3 (King et al., 2011)	Bosentan (dual)	Time to disease progression	Ineffective. Restricted to patients with diagnosed IPF.
MUSIC (Raghu et al., 2013b)	Macitentan (dual)	Forced vital capacity	Ineffective.
ARTEMIS-IPF (Raghu et al., 2013a)	Ambrisentan (ET_A)	Time to disease progression	Ineffective. Halted prematurely due to adverse effects.
Skin			
RAPIDS-1 (Korn et al., 2004)	Bosentan (dual)	Numbers of new digital ulcers	Effective in preventing the formation of new ulcers.
RAPIDS-2 (Matucci-Cerinic et al., 2011)	Bosentan (dual)	Numbers of new digital ulcers. Time to healing.	Effective in preventing the formation of new ulcers. Inefficacious in diminishing time to healing.
Liver			
Tripathi et al. (Tripathi et al., 2006)	BQ-788 (ET_B)	Hemodynamics	Early cirrhosis patients. Incapable of modifying portal hypertension.
Kidney			
ASCEND (Mann et al., 2010)	Avosentan (ET_A)	Proteinuria, end stage renal disease and death	Effective in reducing proteinuria in the short term. Prematurely halted due to adverse effects.
ASCEND follow up (Kohan et al., 2011)	Avosentan (ET_A)	Proteinuria	Kidney disease patients receiving RAS inhibitors. Effective in reducing proteinuria.
Dhaun et al. (Dhaun et al., 2011)	Sitaxsentan (ET_A)	Proteinuria, blood pressure and arterial stiffness	Effective in reducing proteinuria, blood pressure and arterial stiffness.
Heart			
EARTH (Anand et al., 2004)	Darusentan (ET_A)	Left ventricular remodeling	Ineffective.
Prasad et al. (Prasad et al., 2006)	Enrasentan (dual)	Left ventricular remodeling.	Ineffective.

of skin-related symptoms of fibrotic disorders, it should be taken into consideration that DU and RP are particular forms of vasculopathies, and that the efficacy (or the lack of it) of ET receptor blockers on these pathological processes have been attributed to a vascular effect, rather than due to a broader anti-fibrotic action (Steen et al., 2009). Further experimental and clinical studies are needed to conclude whether the blockade of ET signaling may become an effective therapeutic tool for the treatment of skin fibrosis in a more general context.

Liver

Damage to the liver, as occurs under chronic alcoholic ingestion, viral infection or mechanical bile duct obstruction, among other pathological conditions, induces a fibrogenic response intended to maintain organ integrity when extensive necrosis or apoptosis exists. Upon prolonged injury, fibrosis can progress towards excessive scarring and organ failure, as in liver cirrhosis (Friedman, 2008). Activation of hepatic stellate cells remains a central event in liver fibrosis, though there exist solid evidences supporting additional sources of matrix-producing cells including bone marrow-derived circulating cells, portal fibroblasts, and even epithelial–mesenchymal transition from both hepatocytes and cholangiocytes. ET-1 has been consistently reported to be produced by the liver, shifting the cell source of its biosynthesis from sinusoidal endothelial cells to stellate cells during liver injury (Rockey et al., 1998). In fact, ET-1 action on stellate cells is associated with enhanced proliferation, cell spreading, contractility, and also fibrogenesis, all features of the activated (myofibroblast-like) phenotype (Khimji and Rockey, 2011). Further, inhibition of ET signaling with ET receptor antagonists reduces the hepatic fibrogenic response in liver disease experimental models (Feng et al., 2009; Rockey and Chung, 1996). These preclinical studies should have indeed encouraged the testing of ET receptor blockers in cirrhosis patients. However, early clinical studies evaluating their efficacy to treat pulmonary arterial hypertension soon revealed a significant hepatotoxicity, an observation that has hampered the precise analysis of their effect in liver disease (Humbert et al., 2007). Nevertheless, a very few studies in the context of cirrhosis have been done, such as the one testing specific ET_A and ET_B antagonists on hemodynamic effects in early cirrhosis patients (Tripathi et al., 2006). It is clear that in this particular pathological context, new molecules displaying less or no liver toxicity should be developed and tested in clinical studies, looking not only at hemodynamics but also directly at fibrotic effects by using, for instance, FibroScan® (Sandrin et al., 2003).

Kidney

The kidney is a complex organ in which functionality depends on a number of different cell types. The glomerulus, the functional filtration unit of the kidney, is composed of two cell types, the fenestrated endothelial cells and the podocytes, separated by the glomerular basement membrane, essentially a collagen IV-based barrier. Additionally, supporting the glomerular capillaries are specialized pericytes known as mesangial cells, a contractile cell type with the ability to produce and deposit ECM components. Fluid filtered through the glomerulus is collected in the renal tubule, a duct divided in different functional portions in which epithelial cells play an important role in urine formation. Whether started as glomerular, tubular or renovascular damage, insults to the kidney, such as toxins, ischemia, viral or bacterial infection, eventually converges into common renal histological and functional alterations affecting most renal structures, which lead to progressive and generalized fibrosis and glomerulosclerosis, both conditions characterized by enhanced ECM deposition. The origin of myofibroblasts in the kidney has been a matter of intense debate and remains somewhat controversial. Until very recently, it was widely thought that injured epithelial cells served as a primary source of myofibroblasts through an EMT process, an assumption based on the proclivity of epithelial cells to acquire a mesenchymal phenotype in vitro (Ivanova et al.,

2008). However, recent research applying state-of-art fate mapping methodologies have revealed that pericytes and resident fibroblasts are the major, if not the only, source of myofibroblasts in animal models of chronic kidney disease (Campanholle et al., 2013).

ET-1 has been described to be an important factor in renal pathophysiology, and it is reported to influence different cell types. For example, podocytes have been shown to express ET-1 and ET receptors, and also respond to increased ET-1 levels with cytoskeletal remodeling and enhanced protein permeability (Fligny et al., 2011; Morigi et al., 2006). Glomerular endothelial cells are probably the main source of ET-1 within the glomerulus, and this production is increased in chronic kidney disease (Herman et al., 1998; Lehrke et al., 2001). Mesangial cells produce also significant amounts of ET-1, and interestingly, they synthesize and deposit more matrix upon stimulation with ET-1 (Mishra et al., 2003; Sakamoto et al., 1990). As a vasoconstrictor factor, it has been long appreciated that ET-1 contributes significantly to the control of blood pressure, a role implicating renal ET-1 production. Nevertheless, increasing experimental evidences indicate that the actions of ET-1 linking to renal disease are mostly independent of hypertensive effects (Dhaun et al., 2012). In the particular case of fibrosis, overexpression of ET-1 in transgenic mice has been reported to promote collagen deposition with subsequent loss of kidney function, an action not involving hemodynamical effects (Hocher et al., 1997). In fact, ET receptor antagonists have been shown to have beneficial effects in several models of renal disease, including hypertensive or diabetic nephropathy, and glomerulonephritis, with significant reduction in the accumulation of matrix components (Dhaun et al., 2012). Again, in spite of these promising observations, clinical studies are scarce and inconclusive. For example, the ASCEND trial evaluating the effect of avosentan, an ET_A receptor antagonist, on diabetic nephropathy was prematurely terminated due to adverse cardiovascular effects, likely being attributed to significant fluid retention, although partial results showed a significant reduction in albuminuria (Mann et al., 2010). A follow-up study with lower doses of avosentan in patients receiving renin–angiotensin system inhibitors, as well as a recent trial using sitaxsentan (specific ET_A), showed indeed beneficial effects on proteinuria, while lacking moderate detrimental actions (Dhaun et al., 2011; Kohan et al., 2011). Definitely more clinical studies are needed to clarify the potential benefit of ET blockade in chronic kidney disease, not only looking to proteinuria or blood pressure, but also to the extent of renal fibrosis.

Heart and vasculature

As mentioned above, vascular alterations such as RP or DU precede the development of certain fibrotic disorders, such as SSc or IPF (Lagares et al., 2012a; Trojanowska, 2010). Early vasculopathy in these diseases is frequently associated with significant vascular fibrosis (seen as reduced lumen diameter and arterial wall thickening), which somehow is transmitted to the surrounding tissue, lung or skin. Therefore, the vasculature is a major player in the development of fibrosis, supporting the differentiation of matrix-producing myofibroblasts, either by endothelial-to-mesenchymal transition or from pericytes. Taking this into consideration and also the fact that the main source (and likely also the main target) of ET-1 is the vascular system, it is not surprising that ET-1 system influences the synthesis and deposition of matrix components in the vasculature. Thus, mice genetically modified to overexpress ET-1 in the endothelium show enhanced ECM deposition, resulting in structural remodeling of small arteries, an action that occurs without elevation of blood pressure, but can be greatly potentiated by salt loading-induced hypertension (Amiri et al., 2010; Amiri et al., 2004). In agreement with these results, the development of cardiac fibrosis and hypertrophy in mouse models is impaired in mice with vascular endothelial cell-specific ET-1 deficiency (Adiarto et al., 2012). Cardiac fibrosis as a consequence of an ischemic injury or upon pressure/volume overload is a pathological event of important clinical

interest. In addition to its capacity to stimulate cardiomyocyte hypertrophy, ET-1 has been shown to induce matrix protein synthesis as well as proliferation in cardiac fibroblasts, both being crucial events in the fibrotic heart response (Guarda et al., 1993; Piacentini et al., 2000). ET antagonism has been reported to attenuate myocardial fibrosis in animal models of heart disease (Ammarguellat et al., 2001). Administration of bosentan to patients with severe heart failure resulted in hemodynamic and cardiac benefits (Sutsch et al., 1998). These findings have stimulated a number of randomized controlled clinical trials examining the effects of ET receptor antagonists on coronary artery disease and heart failure that have not given a clear positive result, and in many studies even a harmful effect, in most cases attributed to enhanced fluid retention. The list of trials is long (for a recent review, see Kohan et al., 2012) and its detailed description is beyond the scope of this review article. Focused only on those assays having left ventricular (LV) remodeling as primary end-point, assuming that LV remodeling might be considered as an index of fibrosis progression, which is not totally true as cardiomyocyte hypertrophy importantly contributes to this phenomenon, the administration of darusentan as in the EARTH (Endothelin A Receptor antagonist Trial in Heart failure) study, or of enrasentan (a dual ET_A/ET_B antagonist) did not show any favorable effects, despite improved hemodynamics (Anand et al., 2004; Jugdut et al., 2013; Prasad et al., 2006). Again, the great expectations and the tremendous effort on preclinical and clinical trials have been without reward (Kolettis et al., 2013).

Conclusions and future perspectives

Accumulating evidence from cellular and animal data indicate that ET-1 is an important contributor of tissue fibrosis. Human studies also suggest that ET-1 may also be relevant in fibrotic disorders, such as SSc and IPF. Thus, ET-1 emerged as a potential target for pharmacological intervention in the treatment of fibrosis. Nevertheless, the results of a number of clinical trials examining the efficacy of ET receptor antagonists in the context of these diseases did not achieve the primary objectives, a reduction in morbidity/mortality, although they showed a tendency towards those goals (King, 2008). Only bosentan has been approved for use, and in this case, only for one particular case: digital ulceration associated to SSc. Significant liver toxicity or fluid retention has hampered the development of ET receptor antagonists as effective therapeutic agents. Additionally, other factors already mentioned in this review, such as the complex nature of the fibrotic disease, involving multiple factors, or alternative plausible explanations, such as the establishment of no-return points in the onset of fibrosis beyond which the pharmacological intervention offers little or no beneficial effects, might also contribute to the lack of positive results. This should not discourage current and future investigation in the field. Refinement in the toxicological profile of ET blockers, together with a careful selection of dosage and patient recruitment should be implemented in future studies, as well as the opening of this type of drugs to yet unexplored areas, such as the fibrotic response upon trabeculectomy in glaucomatous eyes (Georgoulas et al., 2008). Nevertheless, in any circumstance, an increasing research effort must definitely be directed to the dissection of the complex physiology of the ET-1 system by using both in vitro and in vivo approaches, particularly in the initial stages of the fibrotic response.

Conflict of interest

The authors declare no financial competing interest.

Acknowledgments

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