Chapter 14

Targets for Pharmacological Modulation of Cardiac Fibrosis

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1. Introduction

The heart consists of the myocytes to produce force, the extracellular matrix to provide structural support and the blood vessels to supply nutrients and remove waste products. Studies on the mechanisms of cardiovascular disease, especially heart failure, have traditionally emphasised either deficiencies in myocyte function as the major cause of heart failure or inadequate perfusion as the major cause of ischaemic disease, for example atherosclerosis leading to coronary heart disease and myocardial infarction. The interest in the role of the third component, the extracellular matrix, in cardiovascular disease is much more recent and has emphasised the role of the collagens. The studies of Karl Weber and his colleagues [1-4] have played a key role in the realisation that excessive interstitial and perivascular collagen deposition, termed reactive fibrosis in contrast to reparative fibrosis (scar formation), is a critical component of cardiac remodelling in cardiovascular disease. Thus, Weber [4] argues that it is not the quantity but rather the quality of the myocardium that accounts for ventricular dysfunction in hypertension, the major risk factor for heart failure. This argues strongly that the extracellular matrix is a dynamic, rather than a static, component of the heart. This review will mention the biochemical processes leading to the synthesis and removal of collagens in the heart as the basis for understanding the targets for pharmacological intervention. The major emphasis of this review will be the drugs that may alter these biochemical processes involved in fibrosis. The ultimate aim of therapy with these drugs is to prevent or reverse deficits in cardiac function by controlling or reversing fibrosis to improve the quality of the myocardium.

The extracellular matrix is critically important in the normal function of the heart. This structural and protective framework of the heart connects myocytes, aligns contractile elements, prevents overextending and disruption of

myocytes, transmits force and provides tensile strength to prevent rupture [1, 2]. It contains the fibrillar collagens type I and III, which constitute about 80% and 12 % respectively of the total cardiac collagen content [2], and fibronectin [5]. Collagen I is a heterotrimer that provides tensile strength from parallel rod-like fibres 50-150 nm in diameter. Collagen III is a homotrimer forming a fine network of fibrils. Fibronectin serves as a bridge between cells and the interstitial collagen network to influence cell growth, adhesion, migration and wound repair [5]. Excessive collagen deposition increases cardiac stiffness (or impairs cardiac compliance) and enhances the risk of adverse cardiovascular events such as diastolic and systolic ventricular dysfunction, myocardial infarction, heart failure, and arrhythmias [1, 4, 6].

Since fibrosis is an excessive accumulation of collagen, decreasing collagen synthesis or increasing collagen breakdown are the only ways to prevent or reverse this process. Understanding the complex process of collagen synthesis and degradation is therefore essential to utilise the possible points of attack of pharmacological therapy. Most collagen synthesis in the heart occurs in the fibroblasts. Using collagen I as an example, the mRNAs for the pro-α1 and pro-α2 chains are translated into prepro-α polypeptide chains in the nucleus that are extruded into the endoplasmic reticulum where a signal sequence is removed to give the pro-α chain. Selected proline and lysine residues are then hydroxylated in the presence of molecular oxygen and a reducing agent such as ascorbate. Some lysine residues are glycosylated with glucose or galactose. Three α-chains assemble and disulphide bonds form with the triple helix forming by zipper-like folding. This procollagen molecule is secreted from the Golgi vacuole into the extracellular space where N- and C-terminal propeptides are cleaved by procollagen peptidases. The resulting triple helix collagen molecule then undergoes self-assembly into the fibrils. In the long-term, collagens form glucose-dependent cross-links, the advanced glycation end-products (AGEs) [7].

Mature collagens are cleaved into two unequal fragments by matrix metalloproteinases (MMPs), especially MMPs-1, -2 and -8 (collagenases), produced as proMMPs by fibroblasts before activation by proteases including other MMPs; these fragments are then susceptible to cleavage by other proteases. There are at least 20 different MMPs which includes the collagenases, gelatinases, stromelysins and membrane-bound enzymes. MMP activity is regulated at several levels: by altering synthesis with growth factors, cytokines and corticosteroids, by regulating the activation of the inactive precursors such as the procollagenases and by blocking the enzyme by tissue inhibitors of metalloproteinases (TIMPs) [8]. These processes are altered by common cardiovascular diseases such as heart failure [9]. Fibronectins, like collagens, are degraded by MMPs, especially MMP-3 and -9 which are also inhibited by TIMPs [8].

This summary of collagen deposition and removal indicates that there are many possible targets for pharmacological intervention and some of these interventions are summarised in Figure 1.

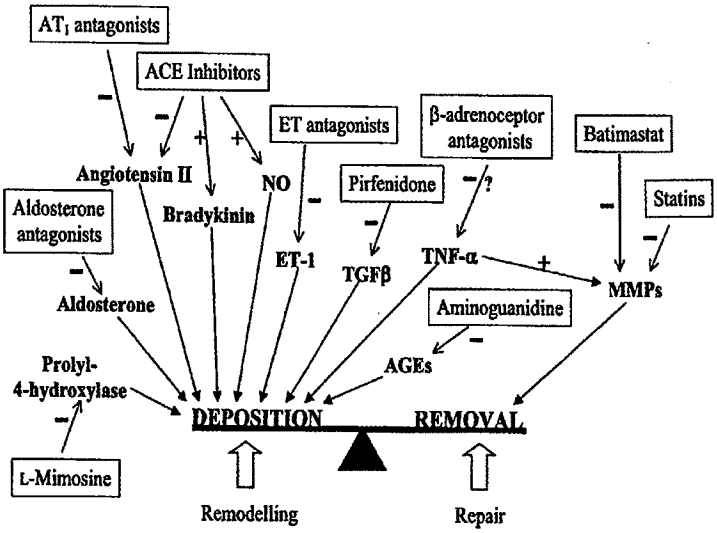


Figure 1. Some of the possible mechanisms to alter collagen deposition and removal in the heart, together with compounds shown to reduce fibrosis probably through these mechanisms.

Most antifibrotic compounds act primarily to decrease collagen expression by fibroblasts (inhibitors of the renin-angiotensin-aldosterone system, endothelin antagonists, calcium entry blockers, NO) or to modify cytokines such as transforming growth factor-β1 (TGF–β1) or tissue necrosis factor-α (TNF-α). Less researched targets include inhibitors of prolyl hydroxylase and lysyl oxidase, activators or inhibitors of MMPs, and modulators of AGE formation. A wide range of chemically unrelated structures has been shown to possess antifibrotic actions; some of the compounds are listed in Table 1.

Since these compounds have many physiological actions through receptors, ion channels, and cytokines distributed throughout the body, it is no surprise that they often have multiple actions on the cardiovascular system and possibly multiple points of attack on collagen synthesis and degradation.

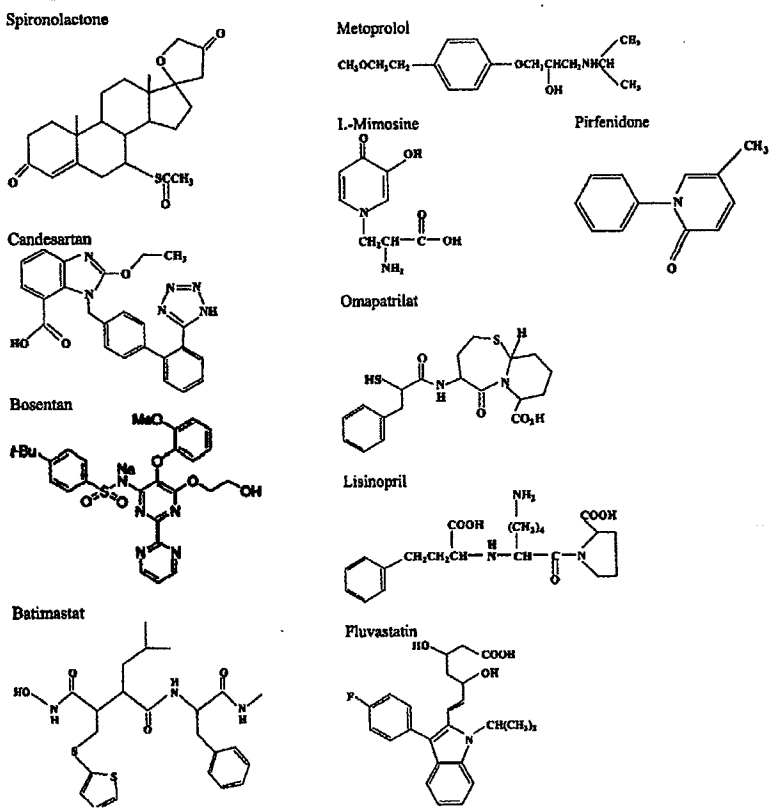
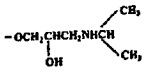
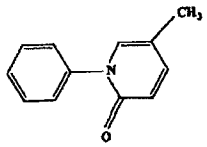


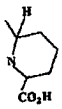
Figure 2. Compounds known to possess antifibrotic actions.

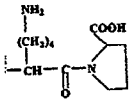
Experimental studies have induced cardiac fibrosis, usually in rats and mice, as a consequence of chronic hypertension, myocardial infarction, or gene deletions or additions. Most commonly used are the rat models of hypertension, in particular the ageing Spontaneous Hypertensive Rat (SHR) [10] or models with an altered renin-angiotensin-aldosterone system (for example, activation with renal hypertension or chronic angiotensin II infusions [11] or suppression in the DOCA-salt hypertensive rat [12]). Uncontrolled chronic hypertension, in both humans and SHR, leads to advanced hypertensive heart disease resulting in heart failure in the final quartile of life [10, 12, 13]. The SHR is widely used as an experimental model to mimic the progression of human essential hypertension although these rats are hypertensive in early adulthood, unlike most



Pirfenidone







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human hypertensive patients [12]. With this early development of hypertension, SHR hearts develop left ventricular hypertrophy accompanied by pathological cardiac remodelling observed as perivascular and interstitial fibrosis [10, 12, 14]. Since these models are also hypertensive and develop cardiac hypertrophy [10, 12, 13], antifibrotic actions may occur together with decreases in blood pressure and cardiac mass, making it harder to define selective antifibrotic effects. Early cardiac remodelling following myocardial infarction includes an increased collagen synthesis and deposition. Myocardial infarction is commonly modelled in rodents by acute ligation of the left coronary artery. This procedure will induce scar formation and heart failure in the relatively short period of 4-8 weeks, unlike the ageing SHR.

In addition, an increasing number of studies are using the techniques of molecular biology, especially cardiac-restricted gene expression, to define the actions of particular genes or products. One limitation of this approach is that cardiac fibrosis developing in humans as a chronic response to cardiovascular damage is unlikely to be the result of a defect in a single gene. Chronic cardiovascular disease is probably multigenic in cause, producing changes in the systems that control the cardiovascular system, especially the renin-angiotensin-aldosterone system, and in endothelial function, especially in the role of producing modulators such as endothelin and NO. Thus, many antifibrotic compounds target the mediators of these systems, such as angiotensin II, aldosterone, endothelin, and NO, rather than a single gene.

2. Inhibition of the Renin-Angiotensin-Aldosterone System

2.1 Inhibiting responses to angiotensin II

The chronic therapeutic management of cardiovascular symptoms in patients with hypertension, heart failure, myocardial infarction and diabetes relies on inhibition of the renin-angiotensin-aldosterone system [15, 16]. Classically, this system consists of liver-derived angiotensinogen being cleaved firstly by kidney-derived renin to inactive angiotensin I and then by angiotensin converting enzyme (ACE) during passage through the lungs to the active octapeptide, angiotensin II, which causes release of aldosterone from the adrenal glands. Organs such as the heart and brain may contain an intrinsic renin-angiotensin system independent of the circulating system. Angiotensin II acts on selective receptors, mostly of the angiotensin receptor Type 1 (AT1) subtype, to effect potent vasoconstriction, growth mediation and stimulation of collagen production; aldosterone is important in electrolyte control by the kidneys and also contributes to fibrosis by actions on non-epithelial cells. The actions of angiotensin II can be reduced by inhibition of its formation by ACE using

compounds such as perindopril and enalapril, or by selective AT1 receptor antagonists such as irbesartan and candesartan. Aldosterone responses can be selectively antagonised by spironolactone.

Cardiac fibrosis characterises chronic cardiovascular diseases and the role of the renin-angiotensin-aldosterone system in the cellular process of fibrosis has been well established in both animal models of cardiovascular disease and in humans [11, 13, 17]. Thus, compounds which inhibit the production or actions of angiotensin II and aldosterone should be effective in controlling an increased blood pressure and both hypertrophy and fibrosis (cardiac remodelling). There is much evidence to support the effectiveness of ACE inhibitors, AT1 antagonists, and spironolactone in the control of cardiac remodelling so that angiotensin II could be described as a gatekeeper of the cascade of fibrosis by modifying the effects of many other possible mediators including NO, TGF-β1, eicosanoids, bradykinin, osteopontin, calcineurin, aldosterone and endothelin.

Angiotensin II rather than mechanical load has been shown to be the major stimulus for increased collagen deposition by cardiac fibroblasts leading to fibrosis [18]. In cultured adult cardiac fibroblasts, angiotensin II induced a dose-dependent synthesis of collagen which was selectively inhibited by the AT1 receptor antagonist, losartan, and unaffected by an angiotensin receptor type 2 antagonist [18, 19]. Local generation of angiotensin II and the novel idea of recruitable ACE have been proposed as mechanisms of local tissue repair. ACE binding density is low in the normal myocardium but high density binding is found in valve leaflets co-localized with angiotensin II and TGF-β1 receptors [20]. Valve leaflets are highly active in the remodelling of their structure particularly in the regulation of type I collagen. In an experimental model of myocardial infarction, high-density ACE binding was evident by day seven at the site of injury [20, 21]. Over the next eight weeks, ACE binding density continued to increase at the site of infarct as well as in other areas of the left ventricle remote to the injury [20, 21]. The recruitable ACE responsible for cardiac fibrosis following myocardial infarction was bound to macrophage and myofibroblast cell membranes and regulated local concentrations of angiotensin II involved in tissue repair [20]. ACE inhibition and AT1 receptor antagonism modulate fibrosis following myocardial infarction. Captopril, enalapril and losartan attenuated infarct size and expansion and inhibited the increase in hydroxyproline levels or collagen found at the infarct site [20, 22, 23].

The DOCA-salt rat is an established model of experimental hypertension characterised by extensive perivascular and interstitial cardiac fibrosis despite a suppressed renin-angiotensin system [24]. However, suppression of the renin-angiotensin system with captopril (100 mg/kg/day, ACE inhibitor), candesartan (2 mg/kg/day, AT1 receptor antagonist), or spironolactone (50 mg/kg/day, aldosterone antagonist) reversed and prevented further remodelling by reducing

collagen expression and deposition [25]. Both captopril and spironolactone also decreased myocardial stiffness without decreasing systolic blood pressure or the degree of cardiac hypertrophy [25].

Most studies with SHR have investigated changes in young adult male rats where there is minimal cardiac fibrosis. As an example, 14 week old male SHR treated with the ACE inhibitor lisinopril (15 mg/kg/day for 12 weeks) showed reversal of the hypertrophy, fibrosis and hypertension with improved functional indices [26]. However, the reversal of established fibrosis in aged experimental models with mild to moderate cardiac failure is more relevant to the treatment of the human condition. Several studies have now examined reversal of existing fibrosis or the prevention of additional fibrosis in ageing SHR. Treatment of male 78 week old SHR with chronic hypertension, advanced ventricular hypertrophy and severe fibrosis with lisinopril (20 mg/kg/day) for 8 months normalised systolic blood pressure, reversed ventricular hypertrophy, attenuated fibrosis possibly by activating MMP-1 and improved diastolic stiffness [14]. Male 65 week old SHR treated with enalapril (30 mg/kg/day) for 12 weeks also showed decreased systolic blood pressure, left ventricular mass, and collagen content as well as improved coronary haemodynamics [27]. Treatment of male 12, 18 and 21 month old SHR with captopril (2g/L in the drinking water) until the age of 24 months prevented the characteristic decrease in the expression of alpha-myosin heavy chain and increase in the expression of pro-αI collagen and TGF–β1 [28] and restored inotropic responsiveness to β-adrenoceptor agonists [29]. Hypertension, hypertrophy, necrosis and fibrosis were prevented in male stroke-prone SHR treated from 1 month of age with ramipril (1 mg/kg/day) up to 15 months of age [30]. Treatment with the same dose of ramipril starting at 15 months of age significantly extended lifespan from 21 to 30 months, attenuated ventricular hypertrophy and endothelial dysfunction and enhanced NO release although collagen deposition was not measured [31].

Many clinical studies have consistently shown that ACE inhibitors prolong survival in human heart failure [32-34]. One reason could be a reversal of fibrosis leading to an improved systolic and diastolic function and fewer arrhythmias. Reversal of cardiac fibrosis has been shown in 18 patients with hypertension, left ventricular hypertrophy and diastolic dysfunction treated orally for 6 months with the ACE inhibitor, lisinopril (11.4±7.2 mg/day) [13]. This elegant study showed improved left ventricular dimensions and function by echocardiography as well as decreased collagen deposition in endomyocardial biopsies. Control patients received the diuretic, hydrochlorothiazide (45.6±9.8 mg/day), which controlled systolic blood pressure to the same extent as lisinopril but failed to improve collagen content or diastolic function [13]. This study highlights local effects of angiotensin II on cardiac myocytes and fibroblasts in humans that are independent of the blood pressure.

2.2. Inhibiting responses to other mediators: bradykinin, osteopontin, AcSDKP, calcineurin and aldosterone

Since bradykinin is a substrate for ACE, the increase in its concentrations following ACE inhibition could explain some of the positive responses to ACE inhibitors. Myocardial bradykinin concentrations in pigs were lower during pacing-induced heart failure while chronic treatment with the ACE inhibitor, benezaprilat (3.75 mg/day), normalised bradykinin concentrations and cardiac output [35]. Selective activation of bradykinin (B2) receptors produced vasodilatation, inhibition of cell growth, stimulation of NO synthase activity causing enhanced NO production [36, 37], and reductions in collagen I and III gene expression [38]. These decreases in collagen expression by fibroblasts were reversed by pre-treatment with indomethacin, a cyclo-oxygenase inhibitor, and mimicked by administration with beraprost, a stable prostacyclin analogue, indicating that increased bradykinin concentrations enhanced prostacyclin production which resulted in attenuation of collagen gene expression [38]. The role of bradykinin has been investigated using a selective B2 receptor antagonist, FR173657 (0.3mg/kg/day orally), in dogs with tachycardia-induced heart failure [39]. The B2 receptor antagonist worsened diastolic function, suppressed NO synthase and sarcoplasmic reticulum Ca^{2+}-ATPase expression and increased left ventricular collagen expression and deposition indicating that endogenous bradykinin participates in the cardioprotective effects of ACE inhibitors [39]. In contrast, co-administration of angiotensin II (150 ng/kg/min sc) and the B2 receptor antagonist, Hoe 140 (115 ng/kg/min sc), for 14 days completely prevented the reactive fibrosis which is characteristic of increased angiotensin II concentrations [40]. Further, in this study, oral indomethacin (2 mg/kg/day) attenuated perivascular collagen deposition suggesting that inhibition of activated myofibroblasts decreased collagen production [40].

The importance of bradykinin B2 receptors has been further defined using receptor gene knockout mice [41, 42]. These mice developed 5-fold higher myocardial fibrosis than control mice at 180 days of age, both as interstitial and perivascular fibrosis [41]. This collagen deposition was completely absent in knockout mice treated with an AT1 antagonist from conception [41]. However, collagen deposition was not different from control mice in another study of B2 receptor knockout mice of similar age either untreated or 12 weeks after coronary artery ligation [42]. The strains differed in that treatment with an ACE inhibitor or AT1 antagonist reduced the increased collagen deposition following infarction only in the control mice [42].

Another proposed mediator of the actions of angiotensin II is the adhesive glycophosphoprotein, osteopontin, an arginine-glycine-aspartic acid (RGD) containing protein which acts like a cytokine mediating cell adhesion,

chemotaxis, and cell signalling. The expression of osteopontin was increased in the heart of SHR coincident with the development of heart failure [43]. There was an increased early expression of osteopontin in the left ventricle of rats transgenic for human renin, a model of angiotensin II-dependent left ventricular hypertrophy and failure [44]. The lack of osteopontin in a knockout mouse model was associated with an absence of an increased collagen following myocardial infarction and an approximately doubled post-infarction left ventricular chamber dilatation [45]. Angiotensin II stimulated osteopontin and TGF-β1 expression but not collagen I expression in fresh samples of human myocardium, suggesting both osteopontin and TGF-β1 as necessary mediators in the human heart [46]. However, there are as yet no studies demonstrating drug-induced inhibition of osteopontin expression leading to a prevention or reversal of cardiac fibrosis.

Plasma concentrations of the naturally occurring inhibitor of pluripotent haemopoietic stem cell proliferation, N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP) are increased during ACE inhibitor therapy since ACE cleaves AcSDKP to an inactive form [47]. Subcutaneous infusion of AcSDKP (800 μg/kg/day for 6 weeks) in aldosterone-salt hypertensive rats markedly prevented the development of both cardiac and renal fibrosis without affecting blood pressure or organ hypertrophy [47]. Rats treated with AcSDKP also showed fewer proliferating cells, probably fibroblasts, in both the heart and kidney [47]. In cultured rat cardiac fibroblasts, AcSDKP inhibited fibroblast proliferation, blocked endothelin-stimulated collagen synthesis and blunted the activation of p44/p42 MAP kinases [48]. Thus, AcSDKP may participate in the antifibrotic effects of ACE inhibitors by suppressing fibroblast proliferation and inhibiting collagen synthesis. AcSDKP inhibited the proliferation of neonatal rat ventricular fibroblasts and decreased phosphorylation and nuclear translocation of Smad2, a key step in the TGF-β1 pathway [49].

The Ca^{2+}-dependent protein phosphatase, calcineurin, an important signalling pathway component leading to cardiac hypertrophy, was activated following treatment of cardiac myocytes with angiotensin II [50]. Its role in cardiac fibrosis has been tested in Dahl salt-sensitive rats [51, 52], rats with abdominal aortic constriction [53] and aldosterone-salt-induced hypertensive rats [54] using the calcineurin inhibitor, FK506 (0.1 – 1 mg/kg/day). Fibrosis and hypertrophy are induced in the Dahl salt-sensitive rat by a high salt diet. Dosage with FK506 from 6 or 12 weeks of age attenuated the development of fibrosis without changing haemodynamic parameters [51]. Similar effects were measured with the AT1-receptor selective antagonist, candesartan (1 mg/kg/day) [52]. Ventricular calcineurin activity is also increased in the pressure-overloaded heart; treatment with FK506 prevented the increased wall thickening and perivascular fibrosis [53]. Mineralocorticoid excess increased expression of calcineurin and collagen and this was reduced either by AT1 receptor

antagonism with losartan (10 mg/kg/day), or calcineurin inhibition with FK506 (0.5 mg/kg/day), or cyclosporine A (10 mg/kg/day) [54].

Activation of the renin-angiotensin system will also increase circulating aldosterone concentrations. Aldosterone acts through mineralocorticoid receptors on cardiac myocytes and endothelial cells and possibly on fibroblasts to induce both perivascular and interstitial fibrosis [55]. Aldosterone promoted fibrosis independent of blood pressure by activation of the transcription factors, AP-1 and NF-κB, and basic fibroblast growth factor in rats doubly transgenic for human renin and angiotensinogen genes [56]. Treatment with valsartan (10 mg/kg/day), an AT1 receptor antagonist, or spironolactone (20 mg/kg/day), an aldosterone antagonist, reduced both transcription factors and collagen [56]. The RALES (Randomized Aldactone Evaluation Study) clinical trial results have led to a re-evaluation of the role of spironolactone in human heart failure [57]. Low doses of spironolactone reduced the risk of death by 30% and improved the symptoms of heart failure. In a sub-group of RALES patients, high concentrations of serum markers for cardiac fibrosis were associated with poor outcome and these markers were decreased during spironolactone therapy [58]. In 46 patients with transmural infarction, the orally active aldosterone inhibitor, potassium canrenoate (50 mg/day), decreased postinfarction collagen synthesis defined by the serum concentration of the aminoterminal polypeptide of type III procollagen and also attenuated progressive left ventricular dilatation [59]. Increased myocardial expression of aldosterone synthase (CYP11B2) has been shown in the failing human heart and this expression and cardiac fibrosis were decreased in patients on spironolactone and ACE inhibitors [60]. In rats, chronic aldosterone-salt treatment increased blood pressure, ventricular hypertrophy and cardiac fibrosis; spironolactone (10 mg/kg/day) prevented collagen expression and deposition without affecting blood pressure or heart weight [61]. In streptozotocin-diabetic rats, spironolactone treatment (50 mg/kg/day for four weeks starting four weeks after streptozotocin) reversed the increased collagen deposition and also attenuated the increased ventricular stiffness of these rats [62].

Since ACE inhibitors may produce some of their beneficial effects by preventing the breakdown of the endogenous vasodilator, bradykinin, further benefit may be obtained by enhancing other endogenous vasodilators. The vasopeptidase inhibitors such as omapatrilat inhibit both ACE and neutral endopeptidase which further enhances NO and vasodilator prostaglandins and increases natriuretic peptides and adrenomedullin by blocking their metabolism. Omapatrilat (40 mg/kg/day for 10 weeks) significantly reduced both interstitial and perivascular collagen deposition as well as systolic blood pressure in 20 week old stroke-prone SHR [63]. However, a comparison of captopril (160 mg/kg/day) and omapatrilat (40 or 80 mg/kg/day) for 8 weeks starting immediately after myocardial infarction in rats showed that omapatrilat

increased circulating atrial natriuretic peptide concentrations but did not result in further structural or functional improvement compared with captopril [64].

Plasma concentrations of circulating cell adhesion molecules such as vascular adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin may predict adverse outcomes in patients at cardiovascular risk [65] while the chemokine monocyte chemotactic protein-1 (MCP-1) is expressed in atherosclerotic plaques [66]. Treatment with enalapril (10–20mg daily) but not losartan (50-100mg daily) lowered concentrations of cell adhesion molecules and MCP-1 in hypertensive patients [67]. ACE inhibitors reduced VCAM-1 concentrations following myocardial infarction [68], in heart failure [69] and in diabetics with borderline hypertension [70]. Since these responses appear selective to ACE inhibitors, the mechanism may involve an increase in NO leading to a decreased inflammatory cell infiltration or oxidative stress. Thus, blockade of angiotensin II formation or responses may lead to many other changes in signalling pathways leading to changes in collagen deposition. Defining the importance of each pathway, especially in different diseases, may lead to disease- or organ-specific control of fibrosis.

3. Modulation of the Endothelial Products Endothelin and NO

Vascular endothelial and smooth muscle cells control vascular tone and cellular proliferation by the synthesis of vasoconstrictors such as endothelin-1 and vasodilators such as NO. Endothelin-1 activates specific ET_A and ET_B receptors and both ET_A-receptor selective and non-selective antagonists have been shown to improve haemodynamics and symptoms in patients with congestive heart failure [71]. ET_A receptors mediate vasoconstriction; ET_B receptors may produce vasodilatation through release of NO. Non-selective endothelin receptor blockade with bosentan (100 mg/kg/day for 9 months) improved survival and decreased cardiac fibrosis, hypertrophy and dilatation in rats with heart failure following coronary artery ligation-induced myocardial infarction [72]. In the DOCA-salt hypertensive rat, bosentan (100 mg/kg/day for 6 weeks) decreased perivascular and subendocardial fibrosis with minimal effects on blood pressure showing that the different components of remodelling are controlled independently [73]. The ET_A-receptor selective antagonist, A-127722 (30 mg/kg/day), prevented the TGF-β1-dependent increase in cardiac collagen deposition induced by endothelin-1 over-expression in the heart also in the DOCA-salt hypertensive rat [74]. This study showed a separation of antifibrotic effects from effects on blood pressure or hypertrophy since systolic blood pressure was decreased to a small extent while hypertrophy was unchanged [74], a result also shown with renin-angiotensin-aldosterone system blockade in this model [25]. ET_A receptor stimulation stimulated collagen accumulation in infarct tissue in the rat since treatment with the selective

antagonist, LU 135252 (30 mg/kg/day), decreased collagen and TGF-β1 gene expression and collagen deposition [75]. However, infarct expansion was increased and systolic function decreased when treatment was started 3 hours after coronary ligation [75]. Treatment with the non-selective antagonist, SB 209670 (6.25 mg/kg twice daily for 26 days starting 48 hours after coronary artery ligation), caused further dilatation of the left ventricle without changing collagen deposition or cross-linking, indicating that early intervention with endothelin antagonists may be harmful [76].

Nitric oxide (NO) is produced by endothelial cells from L-arginine by the action of NO synthase and regulates vascular tone, cardiac contractility, myocardial relaxation, diastolic function, and platelet aggregation [77, 78]. Compounds which release NO such as bradykinin negatively regulated cardiac fibroblast function to decrease collagen I and III expression, probably by increasing intracellular cGMP concentrations [79]. In addition, the NO donor DETA NONOate (100 μM) but not bradykinin decreased proliferation of fibroblasts [79]. In cultured rabbit vascular smooth muscle cells, NO-generating compounds such as S-nitroso-N-acetylpenicillamine and sodium nitroprusside showed reversible, haemoglobin-sensitive inhibition of collagen synthesis, implicating NO release, without damage to the cells [80]. Thus, NO from the endothelium may inhibit local collagen production in the heart and blood vessels. Further, NO suppresses the formation of plasminogen activator inhibitor-1 (PAI-1), which is critical in controlling endogenous fibrinolytic activity and also impairs matrix degradation [81, 82]. Genetically PAI-1-deficient mice treated with the NO synthase inhibitor, L-nitroarginine methyl ester (L-NAME), were protected against the development of coronary perivascular fibrosis indicating that inhibition of vascular PAI-1 activity may prevent fibrosis [83].

Both NO and the renin-angiotensin system are key regulators of vascular tone and there is significant cross-talk between these modulators. NO inhibited angiotensin converting enzyme activity and down-regulated AT1 receptors while angiotensin II stimulated NO synthesis and release [84]. In hypertensive humans, the ACE inhibitor enalapril dose-dependently increased serum nitrate/nitrite concentrations, indicating an increased NO production [85]. This mechanism could provide a mechanism whereby ACE inhibitors selectively suppress fibrosis by increasing NO in contrast to AT1 receptor antagonists which increase angiotensin II concentrations. On the other hand, AT1 antagonists could be more effective than ACE inhibitors in disease states with endothelial dysfunction, such as diabetes and hypertension.

Since NO has a very short half-life and its precursor, L-arginine, is orally active, the importance of NO in fibrosis may be tested by determining changes in cardiac structure and function following chronic L-arginine treatment. In male 12 month old SHR, 6 month treatment with L-arginine (1.2g/l

in drinking water) reduced arterial pressure, peripheral resistance, left ventricular mass and collagen content and improved coronary haemodynamics [86]. Oral L-arginine treatment (2.25% in drinking water) reduced the cardiac pathology of myocarditis and improved survival in a mouse model of encephalomyocarditis virus-infected mice probably by reducing cellular infiltration and myocardial necrosis [87].

The relevance of NO has been further shown by studies in which NO production is markedly decreased using NO synthase inhibitors such as L-NAME. Deficiency of NO following chronic oral administration of this L-arginine derivative to rats induced hypertension, cardiac hypertrophy and fibrosis [88-91]. Inhibition of the synthesis of NO induces many changes that may be therapeutic targets to prevent the cardiovascular remodelling, specifically to inhibit fibrosis. In vivo, chronic inhibition of NO synthesis led to an upregulation of cardiac angiotensin II receptors [92]. Administration of candesartan, an orally active selective AT1 receptor antagonist, reversed the increased blood pressure, left ventricular wall thickness and collagen deposition of L-NAME-treated rats and normalised diastolic stiffness and cardiac function [89]. In L-NAME-treated rats, marked infiltration of leukocytes and fibroblast-like cells into the coronary vessels and myocardial interstitial areas occurred during the first week associated with expression of monocyte chemoattractant protein-1 [93]. The affected areas were replaced after 28 day treatment with vascular and myocardial remodelling. This suggests that early inhibition of inflammation, for example with corticosteroids or non-steroidal compounds, could prevent the subsequent development of fibrosis in this model. Cardiac PAI-1 expression was increased after 7 day L-NAME treatment; this increase was significantly prevented by the ACE inhibitor, imidapril, but not by candesartan, although both compounds inhibited collagen I expression [94]. While the products of the vascular endothelial cells have been primarily considered as modulators of vascular tone and proliferation, it is now clear that these products also control the synthesis and deposition of collagen in the heart.

4. Inhibition of Post-Translational Modifications

Enzymes which play key roles in the intracellular and extracellular maturation of collagen are obvious potential pharmacological targets in the control of cardiac fibrosis [95]. Since hydroxylation of the prolyl residues is a final common pathway in collagen synthesis, inhibition of this enzyme should be a major therapeutic target in reducing collagen production. Few studies have investigated prolyl 4-hydroxylase inhibitors but these have shown the potential of such compounds to prevent myocardial fibrosis and improve cardiac function. In neonatal rat cardiac fibroblasts, ascorbate deficiency led to decreased rates of prolyl hydroxylation without reducing procollagen mRNA levels [96].

Ascorbate-deficient fibroblasts showed increased intralysosomal degradation of newly synthesised procollagens, increased intracellular accumulation of Type I procollagen and decreased extracellular Type I collagen deposition [96]. The naturally occurring catechol analogue, L-mimosine, inhibited prolyl 4-hydroxylase in adult rat cardiac fibroblasts leading to increased intracellular accumulation of procollagens and diminished extracellular secretion with minimal cytotoxicity [97]. Treatment with L-mimosine also induced the activity of MMP-9 to increase the removal of fibrillar collagens [97]. L-Mimosine also reduced the secretion of hydroxyproline-containing proteins from smooth muscle cells obtained from human primary atherosclerotic and restenotic coronary arteries [98]. However, there are no reports investigating the effectiveness of L-mimosine *in vivo*. Treatment with the orally active prolyl 4-hydroxylase inhibitor, FG041 (100 mg/kg/day starting 48 hours after ligation), in female rats with myocardial infarction following coronary artery ligation prevented the substantial increase in the hydroxyproline/proline ratio in the infarcted hearts [97]. Further, there was partial recovery of left ventricular function as measured by echocardiography and haemodynamic measurements [99].

Procollagens are converted to fibrillar collagens by the removal of domains at the N-terminal by N-proteinases and at the C-terminal by C-proteinases allowing spontaneous self-assembly of the monomers. Thus, inhibition of these procollagen proteinases should block the deposition of collagen. Potent, non-peptide analogues of ornithine-derived sulfonamide hydroxamic acids have been shown to be inhibitors of the C-proteinase [100] although no results on cardiac fibrosis have been published. Peptide inhibitors of procollagen N-proteinase have been described [101] but no pharmacological results have been published. However, this could be an important mechanism to control cardiac fibrosis. Both the activity of procollagen C-proteinase (PCP) and its enhancer protein (PCPE) were stimulated by aldosterone in coordination with collagen production [102]. This recent study has shown that spironolactone prevented the upregulation of PCPE and collagen mRNAs following myocardial infarction in rats [102].

Hydroxylation of lysine is necessary for collagen cross-linking. Inhibition of lysyl oxidase would therefore be expected to alter collagen distribution and maturation. Treatment with β-aminopropionitrile (10 g/day orally), an active site irreversible inhibitor of lysyl oxidase, decreased left ventricular collagen deposition and collagen cross-linking and decreased myocardial stiffness when administered to normal adult pigs [103]. In rats treated chronically with 17α-methyltestosterone, β-aminopropionitrile prevented the decreased left ventricular compliance indicating that the increased stiffness with anabolic steroids may be due to increased cross-linking by lysyl oxidase rather than increased collagen formation [104]. Further studies are clearly needed to establish whether inhibition of lysyl oxidase prevents or reverses the

chronic changes in cardiovascular disease. The control of post-translational modification of collagens is clearly an underused mechanism to control cardiac fibrosis.

5. β-Adrenoceptor Antagonists and Calcium Entry Blockers

Although hypertension and fibrosis are clearly independent variables as shown by antifibrotic actions without antihypertensive responses to ACE inhibitors, AT1 and endothelin receptor antagonists, it is feasible that other antihypertensive drugs act by mechanisms that additionally regulate collagen synthesis or degradation. Treatment with some β-adrenoceptor antagonists (carvedilol, metoprolol, and bisoprolol but not bucindolol or celiprolol) has been shown to improve survival in patients with heart failure [15, 16]. In patients treated with metoprolol, attenuated cardiac remodelling was shown as decreased left ventricular end-systolic and diastolic volume indices, decreased left ventricular mass index and an improved left ventricular ejection fraction [105]. However, studies on patients have not investigated whether this attenuated remodelling also involves a decreased deposition of collagen. Chronic β-adrenoceptor antagonism may indirectly decrease collagen deposition based on studies on cytokines in patients with dilated cardiomyopathy treated with metoprolol or bisoprolol [106] and on rat models of hypertension and heart failure treated with carvedilol or metoprolol [107-110]. The increased serum levels of interleukin-10, TNF-α and soluble TNF receptors in patients with dilated cardiomyopathy were significantly decreased during chronic treatment with β-adrenoceptor antagonists [106]. Since these cytokines have been implicated in inflammation and fibrosis, decreased serum levels may lead to a decreased collagen deposition in these patients. Similar effects have been reported in rats with increased TNF-α expression following large myocardial infarctions due to coronary artery ligation and attenuation with oral metoprolol administration (average dose 70.7 mg/kg/day) [108].

Animal studies have shown clear evidence of prevention of collagen deposition following administration of β-adrenoceptor antagonists although responses could be independent of β-adrenoceptor blockade. Administration of carvedilol to stroke-prone hypertensive rats on a high salt-fat diet decreased or prevented myocardial remodelling, in particular the increased hypertrophy, hyperplasia, inflammation, fibrosis and microinfarction without reducing blood pressure [107]. A comparison of equivalent β-adrenoceptor blocking doses of carvedilol (nonselective α- and β-adrenoceptor antagonist) and metoprolol (β_1-adrenoceptor selective antagonist) for 11 weeks showed that only carvedilol significantly reduced myocardial collagen in rats after coronary artery ligation-induced infarction [109]. This difference implies that β_1-adrenoceptors are not

involved in a reduction of cardiac collagen. Consistent with these results, carvedilol but not metoprolol or prazosin reduced the increased collagen and fibronectin production in fibroblasts from rats with left ventricular hypertrophy following aortic banding [110]. The combined α- and β-adrenoceptor antagonist, labetalol, normalised blood pressure but did not regress myocardial fibrosis in rats with 8 week renovascular hypertension, in contrast to equieffective antihypertensive doses of the ACE inhibitor, zofenopril, or the calcium channel antagonist, nifedipine [111]. The relevance of antioxidant responses with carvedilol has not been satisfactorily resolved. Another mechanism to explain the limiting of remodelling and diastolic dysfunction with β-adrenoceptor antagonists may be modulation of MMP activity. Dogs infused with angiotensin II and given 48-hour tachycardia pacing showed increased MMP abundance and activity as well as increased chamber stiffness; these changes were prevented by almost complete β_1-adrenoceptor antagonism with atenolol treatment [112] .

Inhibition of voltage-dependent calcium entry into vascular smooth muscle cells is an accepted antihypertensive mechanism. Further, calcium is an important second messenger in myocytes and fibroblasts and is increased by hormones that cause fibrosis, such as angiotensin II and aldosterone. Thus, calcium channel blockade may have a role in preventing or attenuating cardiac fibrosis. Long-term verapamil for 45 weeks in SHR starting at 10 weeks of age decreased blood pressure and heart weight but did not change collagen concentration [113]. However, nifedipine treatment (30 mg/kg/day for 12 weeks) in renovascular hypertensive rats starting 8 weeks after induction significantly reduced left and right ventricular collagen deposition to a similar extent as the ACE inhibitor, zofenopril [111]. Blockade of T-type calcium channels with mibefradil (30 mg/kg/day for 2 weeks) significantly attenuated myocardial fibrosis in rats receiving either angiotensin II or aldosterone infusions [114]. Chronic treatment with mifebradil (10 mg/kg/day for 6 weeks) reduced interstitial and perivascular fibrosis and improved cardiac function following myocardial infarction in rats [115]. These results indicate that preventing calcium influx through calcium channels may be beneficial in reducing collagen deposition in chronic cardiovascular disease in addition to decreasing blood pressure and reducing anginal attacks.

6. Suppression of Autocrine and Paracrine Systems

The failing heart is characterised by the activation of humoral, autocrine and paracrine systems such as the renin-angiotensin-aldosterone, endothelin, and NO systems discussed above. The activity of other autocrine and paracrine factors in regulating the extracellular matrix provides further possible therapeutic targets to control or reverse collagen deposition. The most important

mediators appear to be several pro-inflammatory cytokines such as the interleukins-1β, -6 and -8 and TNF-α as well as growth factors such as TGF-β, especially in cardiac fibroblasts, which are increased during the remodelling that follows myocardial infarction.

Expression of the interleukins-1β and -6 as well as TNF-α and TGF-β increased following coronary artery ligation in the rat heart [116, 117] and the expression of interleukin-1β correlated well with collagen deposition in the non-infarcted myocardium [116]. Treatment with anti-interleukin-1β antibody suppressed cardiac collagen expression and accumulation following myocardial infarction in mice [117]. However, wound healing mechanisms were delayed which led to left ventricular dilatation and an increased risk of ventricular rupture in these antibody-treated mice suggesting that interleukin-1β plays a protective role in the acute phase after myocardial infarction [118]. The role of an increased interleukin expression in the late phase has not been determined nor has the importance of an increased interleukin expression in other models of cardiac fibrosis such as the ageing SHR. One member of the interleukin-6 family of cytokines, leukaemia inhibitory factor (LIF), has multiple effects on collagen synthesis and degradation [119]. In mice cardiac fibroblasts, LIF inhibited differentiation into myofibroblasts, reduced collagen content and also reduced MMP activity [119].

TGF-β is a potent stimulus for matrix deposition by increasing the expression of collagen, decreasing the expression and activity of collagen degrading proteolytic enzymes such as MMP-2 and -9 and enhancing the expression of MMP inhibitors, the tissue inhibitors of MMPs (TIMP-1, -2 and -4) [117, 120-122]. TGF-β1 signalling occurs via ligand-induced heteromeric complex formation of type I and type II serine/threonine kinase receptors and downstream through the Smad protein family [123, 124]. In the mammalian heart, the Smad proteins are divided into the receptor-regulated Smads 2 and 3, the common mediator Smad 4 and the inhibitory Smads 6 and 7. After TGF-β1 receptor activation, the regulatory Smads are phosphorylated and form a dimer with Smad 4 which translocates to the nucleus to regulate gene transcription [123, 124]. Myocardial infarction leads to complex changes in this signal transduction pathway. Expression of TGF-β1 in isolated cardiac nonmyocytes was increased after 1 week before returning to baseline at 6 weeks [117]. In the whole rat heart, TGF-β1 expression was enhanced on day 2 after infarction and remained elevated for 28 days [125]. Smad 2, 3 and 4 proteins were significantly enhanced in border and scar tissues [126] while Smad 7 expression was decreased [127]. However, selective expression of TGF-β1 resulted in atrial but not ventricular fibrosis in transgenic mice, indicating that increased receptors or activating proteins are also necessary [128]. TGF-β1 may also increase the expression of other growth factors such as connective tissue growth factor

(CTGF) that can trigger cell proliferation, adhesion, migration and the synthesis of extracellular matrix [129]. In addition, TGF-β1 suppressed the activity or expression of NO synthase, especially the NOS2 isozyme expressed following stimulation with inflammatory compounds [130], which could also participate in increasing collagen synthesis and deposition. The increased collagen production in rat ventricular fibroblasts in culture may be due to an increased differentiation of fibroblasts to myofibroblasts that have a higher collagen production [131]. Thus, attenuation of these changes should lead to decreased collagen in the heart.

There are many studies showing that suppression or attenuation of the TGF-β1 pathway improves cardiac structure and function. Heterozygous TGF-β1 deficient mice showed decreased age-associated myocardial fibrosis and improved compliance which may have contributed to the improved survival [132]. Blockade of the actions of angiotensin II with the selective receptor antagonist, losartan, normalised Smad 2 and 4 over-expression and these changes were paralleled by modulation of the fibroproliferative events both in post-myocardial infarction rat hearts [133] and in Syrian hamsters at early and late stages of cardiomyopathy [134]. Combined blockade of angiotensin and endothelin receptors reduced TGF-β1 and collagen expression and improved ventricular function [135]. Suppression of an increased TGF-β1 expression by tranilast in hypertensive transgenic rats over-expressing human renin attenuated left ventricular hypertrophy and fibrosis without lowering blood pressure [136]. Daily administration of anti-TGF-β neutralising antibody in rats with pressure overload following aortic constriction inhibited fibroblast activation and subsequently collagen expression and myocardial fibrosis; diastolic dysfunction was reversed without affecting blood pressure, myocyte hypertrophy or systolic function [137].

Suppression of an increased TGF-β1 expression may be the mechanism for the antifibrotic actions of pirfenidone [138, 139]. Pirfenidone may also increase collagen breakdown by reducing the TGF-β1-induced inhibition of the degrading enzymes, the MMPs [140]. During chronic administration, pirfenidone consistently prevented collagen accumulation, for example in bleomycin-induced pulmonary fibrosis in hamsters [141] with attenuation of pulmonary functional deficits [142]. Pirfenidone reversed collagen deposition and reduced cardiac stiffness in streptozotocin-diabetic rats [62] and in DOCA-salt hypertensive rats [143].

In rat cardiac fibroblasts, TGF-β expression and production can be inhibited by hepatocyte growth factor (HGF) [144]. This study tested the role of HGF in cardiac fibrosis in cardiomyopathic hamsters treated with an ACE inhibitor or a selective angiotensin receptor antagonist. Angiotensin II blockade prevented myocardial fibrosis, accompanied by a significant increase in HGF,

implying that local HGF expression may prevent myocardial injury [144]. In a further study, the human HGF gene was transfected into the heart of cardiomyopathic hamsters [145]. After 8 weeks, collagen density was decreased through activation of MMP-1 and inhibition of TGF-β expression; in addition, therapeutic angiogenesis was shown as an increased cardiac capillary density [145].

Cytokine activation is important in cardiovascular disease progression, in particular in heart failure [146, 147]. One of these cytokines, TNF-α, activates specific TNF-α receptors on all nucleated cells in the heart to change myocyte size and viability and up-regulate the different MMPs to induce variable proteolysis of extracellular matrix components. Since an increased TNF-α produces many responses and TNF-α concentrations are only one of many changes in heart failure, it is difficult to determine specific effects on cardiac collagen metabolism following TNF-α suppression in heart failure patients. One promising technique is the use of transgenic mice with over-expression of TNF-α that is restricted to the heart [148]. These mice showed an increase in MMP activity and a decrease in cardiac fibrillar collagen in the early stages followed by a significant decrease in MMP activity and increased collagen content as the mice aged. These changes in the ageing mice were associated with increased levels of both TIMP-1 and TGF-β. The relationship between TNF-α and MMPs has been investigated in dogs with evolving heart failure with 28-day chronic pacing given etanercept [149]. TNF-α block reduced or prevented pacing-induced changes in end-diastolic volume and MMP levels indicating that TNF-α acts by inducing specific MMPs [149]. Recent studies have shown that angiotensin II increased TNF-α expression, probably by a protein kinase C pathway, in the feline heart and in cultured cardiac myocytes [150]. This up-regulation was mediated by the AT1 receptor subtype which may explain why chronic blockade of these receptors reduced circulating TNF-α concentrations.

Despite these studies implicating TNF-α in cardiac fibrosis, there is little experimental or clinical evidence as yet that decreased TNF-α concentrations will decrease cardiac fibrosis and improve cardiac function. Etanercept, a recombinant TNF-α receptor antagonist that functionally inactivates TNF-α, improved ventricular function and remodelling in preliminary trials in heart failure patients [151]. However, reports state that the large RENEWAL clinical trial with etanercept has been prematurely stopped since interim analysis showed no likelihood of a difference between placebo and etanercept [152]. There are no reports measuring possible changes in cardiac collagen metabolism. Several drugs shown to block TNF-α expression such as prednisone or enhance mRNA degradation such as thalidomide or decrease TNF-α concentrations such as pentoxifylline have been shown to be beneficial

in heart failure but their effects on cardiac collagen are unclear or unproven [147].

Brain natriuretic peptide (BNP) may be another locally-produced growth factor that acts as a myocyte-derived counter-regulatory mechanism to cardiac fibroblasts [153]. In this study, mice with targeted disruption of BNP showed marked fibrotic lesions without cardiac hypertrophy or systemic hypertension but with increased expression of ACE, TGF-β1 and pro-α1-collagen [148]. These BNP -/- mice also showed an increased fibrosis with an acute pressure overload, indicating that BNP moderates overload-induced progression of fibrosis [153].

The role of growth hormone and its major mediator, insulin-like growth factor-1 (IGF-1), have received little attention yet chronic excess of growth hormone in humans causes increased interstitial fibrosis as the major histological abnormality of the heart [154]. The major mediator may be an activated renin-angiotensin-aldosterone system. IGF-1 stimulated growth of neonatal rat cardiac fibroblasts which could be inhibited by either ACE inhibition or AT1 receptor antagonism [155]. The renin-angiotensin system is involved in the growth hormone-mediated modification of electrolyte and fluid homeostasis increasing angiotensinogen concentrations and angiotensin receptor density in the liver, kidney, and adrenals of dwarf rats supplemented with growth hormone [156], but similar effects on cardiac receptors have not been reported. Growth hormone has been reported as a treatment for heart failure in small trials, although a larger trial showed no benefit on cardiac structure or function [157]. The potential of growth hormone to worsen cardiac fibrosis should be considered in future trials.

Since cytokines are clearly involved in the progression of cardiac fibrosis and heart failure, the understanding of their multiple roles in the cardiovascular system is essential in understanding the possibilities for altering the progression of cardiovascular disease.

7. Inhibition of Inflammation, Free Radicals and Oxidative Stress

Interstitial fibrosis is accepted as a final common response to chronic inflammation, although fibrosis will become independent of the inflammatory process at some stage. Fibroblast activity and collagen deposition were closely related to the presence of lymphocytes and macrophages in the myocardium of ageing SHR [158]. In renovascular hypertensive rats, the correlation between macrophage density and plasma renin activity indicated that angiotensin II may be the initial signal which mobilised inflammatory cells [159]. Intercellular communication and intracellular signalling which confer an inflammatory

phenotype to arteries have been reviewed [160]. The role of NO in inflammation remains unclear and it has been proposed that the physiological chemistry of NO may account for the differing responses [161]. Prevention of inflammation should prevent reactive cardiac fibrosis if this process is the response to inflammation.

Corticosteroids inhibit the synthesis of pro-inflammatory cytokines such as TNF-α. Chronic methylprednisolone (5 mg/kg/day for 21 days) prevented both interstitial and perivascular collagen deposition in the spared myocardium following myocardial infarction in rats [162]. In addition, baseline left ventricular function was improved by methylprednisolone treatment. These authors also showed that low-dose aspirin (25 mg/kg/day) reduced perivascular collagen deposition but this was not reflected in an improved ventricular diastolic function, possibly because interstitial collagen was unchanged [162]. This dose of aspirin had been shown previously to selectively inhibit platelet thromboxane production and lower plasma thromboxane concentrations without affecting left ventricular dysfunction in rats with myocardial infarction [163]. However, the key question remains whether the fibrotic process can be reversed by inhibition of inflammation.

Inflammation is also an important component of the atherosclerotic process. The major drugs used to reduce endogenous cholesterol biosynthesis to decrease atherosclerosis are the statins, orally active inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase. While many large trials have shown their beneficial effects in the prevention of coronary artery disease, recent evidence suggests that these compounds have important cholesterol-independent effects to restore endothelial function, enhance the stability of atherosclerotic plaques and decrease oxidative stress and vascular inflammation [164]. Many of the putative mechanisms may also lead to a reduction of cardiac collagen deposition, for example decreases in endothelin synthesis, reactive oxygen species, proinflammatory cytokines and MMP expression and secretion [164]. In a rat model of vascular remodelling following aortic banding, fluvastatin prevented the increased formation of superoxide anions and ICAM-1 expression in the aorta that was associated with an enhanced expression of endothelial nitric oxide expression and decreased perivascular fibrosis [165]. These antifibrotic changes probably also occur in the heart. Treatment of mice with coronary artery ligation-induced heart failure with fluvastatin (10 mg/kg/day for 4 weeks) reduced interstitial fibrosis and myocyte hypertrophy while improving left ventricular performance and survival [166]. These benefits were associated with an attenuation of the infarct-induced increase in left ventricular MMPs. Cholesterol-independent protective effects on the heart have been demonstrated in a double transgenic rat model with both the human renin and angiotensinogen genes. These rats develop severe cardiac and renal inflammatory injury as angiotensin II-induced end-organ damage and die at about 7 weeks of age if

untreated [167]. Treatment with oral cerivastatin (0.5 mg/kg/day for 3 weeks) reduced mortality, blood pressure, cardiac hypertrophy, macrophage infiltration, and extracellular matrix (collagen, laminin and fibronectin) deposition [167]. Interstitial fibrosis together with cardiac hypertrophy and left ventricular dysfunction are some of the key characteristics of hypertrophic cardiomyopathy. These characteristics were recapitulated in rabbits with cardiac-restricted expression of β-myosin heavy chain-glutamine 403 (Q^{403}) [168]. In this model, treatment of adult rabbits with simvastatin (5mg/kg/day) for 12 weeks reduced collagen volume fraction and left ventricular mass and also improved left ventricular filling pressures [168]. One possible mechanism was a reduction in the activation of the predominant stress-responsive intracellular signalling kinase, ERK 1/2. These studies clearly show that the cholesterol-independent effects of the statins could be remarkably useful in improving the structure and function of the human heart in chronic cardiovascular disease by decreasing both hypertrophy and fibrosis.

Free radical-mediated cellular damage may be one possible cause of haemodynamic abnormalities leading to cardiac remodelling and dysfunction. Treatment with antioxidants may alleviate this oxidative stress and reduce cardiac damage. Myocardial infarction in rats has been shown to decrease concentrations of vitamin E in the left ventricle and liver and of vitamin A in the liver and kidney; dietary vitamin E supplementation led to an improved haemodynamic function [169]. However, vitamin E supplementation did not prevent cardiovascular events in a large trial of patients at high risk [170]. In mice with myocardial infarction following coronary artery ligation, hydroxyl radical concentrations were increased in the non-infarcted myocardium and the mice showed the symptoms of heart failure [171]. Treatment with the hydroxyl radical scavenger, dimethylthiourea (50 mg/kg/day ip for 4 weeks), attenuated the increased collagen deposition and myocardial MMP-2 activity while left ventricular function was significantly improved [171].

Probucol is an effective cholesterol-lowering compound with potent antioxidant properties. Oral probucol (61 mg/kg/day for 4 weeks) started 24 hours after coronary artery ligation in rats increased scar thickness and decreased cardiac fibrosis without altering ventricular hypertrophy or dilatation [172]. The positive effects on cardiac fibrosis may be due to decreased cardiac oxidative stress and expression of the proinflammatory cytokines, interleukin-1β and -6 [172]. Probucol (61 mg/kg/day for 80 days starting 20 days after infarction) improved left ventricular function in mice with heart failure following coronary artery ligation [173]. Cardiac fibrosis was decreased while left ventricular dilatation and wall thinning were prevented [173]. Oxidative stress may also be one factor in the development of cardiac interstitial fibrosis in renal failure as treatment of rats with subtotal nephrectomy with tocopherol

(vitamin E; 2x1500 IE/kg/week for 12 weeks) attenuated but did not prevent interstitial fibrosis [174].

ACE inhibition may also be an effective antioxidant strategy by decreasing angiotensin II concentrations and increasing bradykinin and NO bioactivity [175, 176]. Oxidative stress begins in the vascular wall (endothelium, smooth muscle and fibroblasts) by enzymes that use NADH and NADPH as substrates for superoxide anion formation [175]. These enzyme systems are activated by angiotensin II and by a decrease in bradykinin levels leading to fibrosis, cell death, and necrosis [175]. Additionally superoxide reacts with NO to inactivate it, decreasing its bioavailability [176]. Overactive superoxide formation is also involved in the vascular pathology of diabetes mellitus [177]. Enalapril prevented oxidative stress in cells from streptozotocin-diabetic rats, inhibiting fibrosis and end-organ damage in the left ventricle, kidney and liver [177]. There is still much more to discover on the interrelationships between inflammation, oxidative stress and cardiac fibrosis.

8. Activation of Matrix Breakdown

The role of the MMPs, a family of at least 20 zinc-dependent enzymes responsible for myocardial matrix degradation, in the progression of cardiovascular disease is now being elucidated. The MMPs are regulated by many growth factors, cytokines and matrix fragments such as the matrikines [178, 179]. In addition, the endogenous physiological inhibitors of the MMPs, the tissue inhibitors of metalloproteinases (TIMPs), can also be regulated. Thus, the progression of the fibrotic process is determined by the interplay of MMPs, their inhibitors, and regulators, all of which may be altered in cardiovascular disease [178]. As an example, myocardial MMP-2 remained inactive during compensated left ventricular hypertrophy in Dahl salt-sensitive rats but was activated during the transition to heart failure [180]. Although TIMPs were also activated, the greater activation of MMP-2 may result in matrix breakdown and the progression of left ventricular dilatation following myocyte slippage. Thus, inhibition of MMPs would be a therapeutic target in the failing heart undergoing ventricular enlargement [181]. The actions of the nonselective MMP inhibitor, batimastat, on cardiac collagen, heart function, and survival have been measured in transgenic mice with cardiac-restricted overexpression of TNF-α [182]. In young mice, batimastat reduced collagen expression but increased insoluble collagen while myocardial hypertrophy and diastolic dysfunction were prevented and survival improved. However, no improvements were measured in old mice with established heart failure. Thus, MMP inhibition may be important in the treatment of heart failure but only early in its development [182]. Treatment for 4 months with PD166793 (5 mg/kg/day) of obese male spontaneously

hypertensive heart failure (SHHF) rats attenuated the ventricular enlargement characteristic of the development of failure although collagen content was unchanged [183]. ACE inhibitors such as ramipril may also alter myocardial remodelling by MMPs in heart failure. In 16 week old SHR with heart failure following occlusion of the left coronary artery, ramipril (1 mg/kg/day for 6 weeks) reduced MMP-2 and collagen type 1 expression and increased TIMP-4 levels. These changes were associated with prevention of left ventricular dilatation, reduction of fibrosis, decreases in left ventricular end-diastolic pressure and mortality and increases in left ventricular pressure [184]. Although MMP inhibitors may be useful in the failing heart, these compounds may increase collagen deposition and ventricular stiffness in the non-failing heart [181].

One difficulty is to decide whether the aim of pharmacological modulation should be MMP activation or inhibition. Activation could be beneficial by allowing the removal of excessive collagen deposits. This is clinically attractive since it is essentially the reversal of an existing disease process, rather than prevention, but collagen removal could also cause progressive ventricular dilatation in the failing heart [179, 182]. Activation of MMPs with the serine protease, plasmin, acutely degraded collagen and decreased the elastic stiffness constant and viscosity constant in papillary muscles from hypertrophied hearts [179]. This study clearly shows that acute removal of collagen improved the function of hypertrophied myocardium.

9. Reduction of Cross-Linking

Collagen cross-linking occurs initially by the Maillard reaction of glucose with the amino groups of proteins to form a chemically reversible Schiff base adduct which rearranges to the more stable but still chemically reversible Amadori product, a ketoamine (Figure 2) [7]. AGEs are formed by further reactions of these Amadori products with amino groups on other proteins to form stable intermolecular cross-links. Several receptors which may mediate the responses of AGE have been identified. The best characterised receptor for AGE, known as RAGE, is increased in diabetes possibly to act as a scavenger and mediate intracellular signalling [7]. One possible signalling pathway leads to oxidant stress and activation of NF-κB to increase the generation of pro-inflammatory cytokines [7]. AGEs have also been shown to increase the expression of MMP-2, MMP-9 and MMP-13 in isolated rat cardiac fibroblasts which may alter cardiac remodelling [185].

Cross-linking of collagen fibres in the heart may be an important mechanism for the increased cardiac stiffness and more relevant than changes in collagen content [186]. This is supported by studies showing that therapeutic

modulation of AGEs either by inhibiting AGE formation or breaking AGE cross-linkages improves cardiac function. The nucleophilic hydrazine, aminoguanidine (pimagedine), probably reacts with the reactive ketoamine Amadori product producing an unreactive product which leads to decreased cross-linking of collagens (Figure 2) [187]. Aminoguanidine has many possible mechanisms of action in the cardiovascular system, including selective inhibition of iNOS, quenching of hydroxyl radicals as well as inhibition of free radical formation, lipid peroxidation and oxidant-induced apoptosis [188].

As an example, aminoguanidine treatment of 13 month old normotensive Sprague Dawley rats for 9 months reduced blood pressure, improved glomerular filtration rate and renal plasma flow while reducing glomerular sclerosis but did not alter oxidative stress, lipid peroxidation, or immunostaining for AGEs, indicating that inhibition of iNOS was the most likely mechanism of action [189]. However, treatment of 6 month old Sprague Dawley and Fisher 344 rats for 18 months prevented the significant increases in AGE accumulation in the heart, aorta and kidney and also prevented age-linked vasodilatory impairment, indicating that interference with AGE accumulation by aminoguanidine may protect against cardiovascular and renal decline in ageing [190]. In normotensive male WAG/Rij rats, treatment with aminoguanidine for 6 months from 24 months of age prevented cardiac hypertrophy and arterial stiffening without changing collagen and elastin content [191]. In streptozotocin-diabetic rats, treatment with aminoguanidine for 4 months prevented both the increase in collagen cross-linking and the increased myocardial stiffness without changing the elevated blood glucose concentrations [192]. More potent inhibitors of the formation and accumulation of AGEs than aminoguanidine have been reported [193, 194]. However, the cardiovascular responses to chronic treatment have not yet been reported.

The breakage of established AGE cross-links is an additional potential mechanism to reverse the chronic effects of an increased collagen deposition, rather than to prevent collagen accumulation. The thiazolium derivative, ALT-711 (phenyl-4,5-dimethylthiazolium chloride), when given to aged dogs at 1 mg/kg daily for one month, reduced left ventricular stiffness by approximately 40% and increased stroke volume index since end-diastolic volume increased [195]. A possible mechanism has been described together with a review of cardiovascular studies of these compounds indicating their potential usefulness in ageing and diabetes [196]. Collagen cross-linking is an integral part of the chronic changes in cardiovascular disease so that either prevention or reversal of these processes holds promise for the improvement of cardiovascular function in chronic diabetes and hypertension.

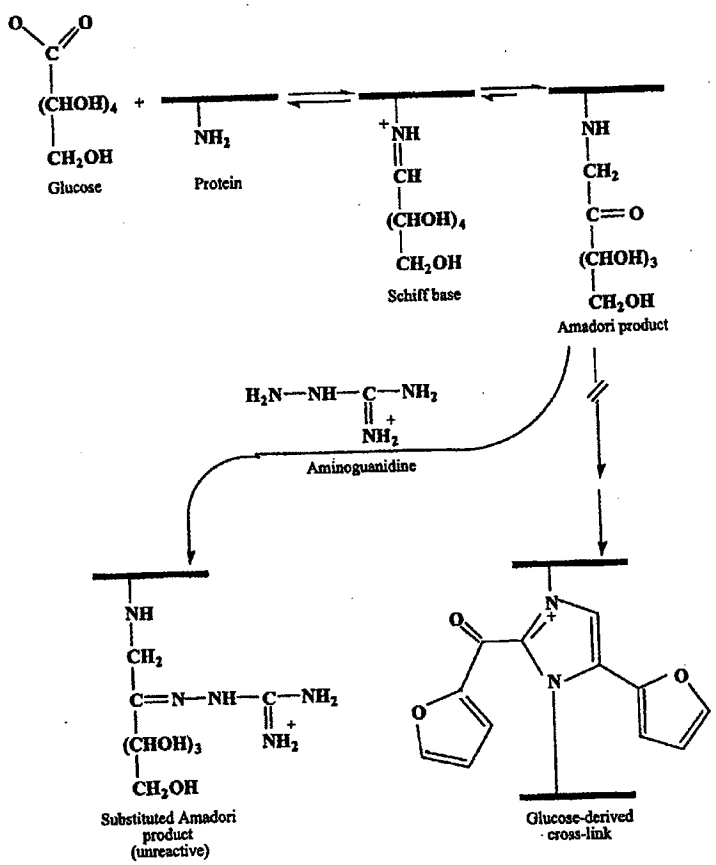
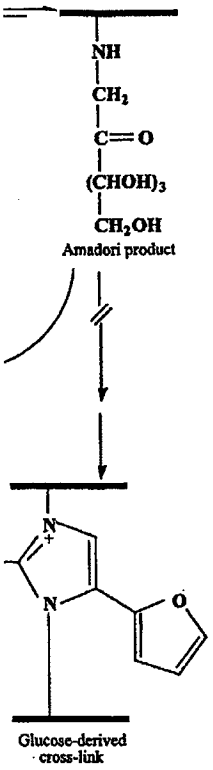


Figure 2. Mechanism of action of aminoguanidine.

10. Summary

These studies argue convincingly that the extracellular matrix, in particular collagen, is a dynamic component of the heart. Collagen deposition and removal are remarkably complex processes but this complexity provides many possible targets for pharmacological intervention. Many compounds, some



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in current therapeutic use especially in patients with hypertension, diabetes, and heart failure, have been shown to alter collagen content. While other compounds are unlikely to become therapeutic tools, they are allowing an investigation into possible mechanisms for the prevention or reversal of fibrosis. More importantly, these studies have shown us that controlling cardiac collagen is not simply a biochemical curiosity since many studies have now shown improvements in the functioning of the diseased heart. While most of these studies are in rodent models of human cardiovascular disease, those studies on humans are also positive. Research into cardiac collagen is still gaining momentum with almost all the studies cited in this review having been published in the last 10 years and many in the last 2-3 years. Thus, pharmacological control of collagen in the heart is likely to become a standard and successful component of the therapy of human cardiovascular disease.

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