Collagen Crosslinking in the Heart: Relationship to Development and Function

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Abstract

The hydroxypyridinium (HP) crosslink is the predominant non-reducible collagen crosslink in heart. HP concentration in left ventricle (LV) increases progressively throughout life, and this increase is thought to reflect the slower turnover of collagenous proteins seen with aging, allowing mature extracellular matrix (ECM) collagen to crosslink more heavily. There are also species differences with higher levels of crosslinking found in the hearts of larger mammals including humans, compared to smaller mammals such as the rat. Marked deviations from normal in the concentration of this crosslink are implicated in a variety of left ventricular hypertrophies and altered ventricular function. Interestingly these deviations may be bi-directional in nature, ranging from an apparent lack of the crosslink in a mouse cardiomyopathy model, to a doubling in HP concentration in viable myocardium post-infarction. This review outlines the major pathway involved in the formation of myocardial collagen crosslinks. Possible mechanisms by which rate of crosslink formation and deposition are regulated will be discussed, and functional implications of altered crosslinking patterns addressed.

Key words: collagen, crosslinking, heart, myocardium, decorin.

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Collagen fibrils and the fibrous matrices they form are stabilized by covalent crosslinks. Normal crosslink formation is essential to the development of functional tissue and organs; abnormal crosslink formation characterizes well-known disorders such as Menke's disease, cuds laxis or forms of Ehlers-Danlos syndrome. Mechanisms by which crosslinks form and implications of both normal and aberrant crosslinking patterns in many tissues have been addressed in several comprehensive reviews [7, 8, 26, 37].

Our understanding of the role covalent collagen crosslinking plays in heart function or in the development of various cardiac pathologies is still in its early stages. Several observations, however, make collagen crosslinking an intriguing focus for investigations involving heart extracellular matrix (ECM). For instance, alterations in crosslink concentrations are associated with lethal pathologies as well as physiological adaptations. Shifts in crosslink concentration can be bi-directional and both elevated as well as depressed levels of crosslinking can be related to heart dysfunction. Finally, crosslink abnormalities affect both systolic and diastolic function of the heart.

Our goal, in this brief review, is to examine the experimental data documenting the nature and extent of collagen crosslinking in heart muscle. Two key questions raised, if not entirely answered, by the available information are: 1) what are the mechanisms regulating crosslink formation and accumulation? 2) what is the role of crosslinking in heart function?

The Structural Arrangement of Myocardial Collagen

The collagen network of the myocardium is arranged hierarchically, in a fashion analogous to the collagen of skeletal muscle [19]. A thin outer sheath of connective tissue, predominantly collagen, encloses the entire myocardium. The connective tissue surrounding bundles of myocytes is perimysium, while that sheathing and interconnecting individual myocytes is endomysium. The composition of the perimysial and endomysial connective tissue depots is predominantly types 1 and 111 collagen [22]. Based on immunohistochernical staining data, the perimysial fraction possesses somewhat less type 111 collagen than the endomysium [I], an observation consistent with the distribution of these collagen phenotypes in skeletal muscle. We are unaware of studies which have compared the amount of collagen or concentration of crosslinks in the perimysial and endomysial fractions of the myocardium; however, in skeletal muscle the bulk of collagen (~ 90%) and most of the non-reducible crosslinking occur in the perimysium [19].

The connective tissue depots of the myocardium possess several unique structural features. In the adult heart, perimysium may exist structurally as a weave of collagen

fibers surrounding bundles of myocytes [4, 28], as tendon-like collagen strands interconnecting the perimysial weave [27] or as coiled perimysial fibers running in the direction of the muscle fibers [27]. Endomysial connective tissue is configured as collagen struts [4] or as a collagen-microthread-lattice [28]. Collagen struts make individual myocyte-to-myocyte and/or myocyte-to-capillary connections. They help maintain constant myocyte length during diastole and capillary and coronary blood during systole [22]. The microthread lattice serves to link collagen struts to each other and to anchor struts and larger collagen fibrils to the myocyte surface [28].

Collagen and Collagen Crosslink Chemistry

To date, 19 different collagen pheiiotypes exhibiting a wide degree of structural and functional diversity have been described. Because the predominant collagens in the myocardium are the banded, fibrillar collagen types I and III this review will focus only on their structures and mechanisms of crosslink formation. The fibrillar collagen molecule consists of three polypeplidc subunits, termed a.-chains, which associate via hydrogen bonding to form a superhelix. Both I and TIT phenotypcs arc characterised by a large central triple helical domain consisting of a repeating (GLY-X-Y) triplet and small non-helical regions at the C- and N-ter-mini, called telopeplides. Collagens are rich in glycine, praline, alaninc and hydroxyproline which contribute significantly to interchain hydrogen bonding [23].

Collagen molecules undergo extensive post-translational modifications. Intracellularly, selected praline and lysine residues are cnzymatically hydroxylated and some hydroxylysines are then glycosylated. Extracellularly, the telopeptide regions are proteolytically processed and the molecules assemble via hydrophobic and electrostatic interactions into a head-to-tail array to form microfibrils. Molecules aggregate laterally into nascent fibrils in a regular fashion with five molecules to the row with each molecule overlapping the next by about one quarter of its length producing a quarter staggered array [23].

Initial fibril orientation is not stable; only non-covalent interactions maintain the association of collagen molecules in the immature fibril. Collagen molecules are free to slide past one another and the immature fiber is subject to proteolysis {collage no lysis) and disruption experimentally by variations in ionic strength or temperature. The high tensile strength of the mature fibril and, thus, its functionality is conferred largely by intermolecular crosslinks [7, 25].

Immediately upon fibril aggregation, selected lysine and hydroxylysine residues are cnzymatically deaminated via lysyl oxidase, creating lysine- or hydroxyly sine-derived aldehyde functions (allysine and hydmxyallysine, respectively). In the fibrillar collagens, i.e., types I and III, four principal crosslinking sites have been reported [7]. Two sites occur toward the N-terminus, one in the telopeptide region (residue number 9N) and Ihe other in the helical region at residue number 87. The second pair of sites occur toward the C-terminus, one at residue number 930 (helical region) and the other at 16 C (teiopeptide region}. These four loci may be the only crosslinking sites in the banded fibrillar collagens, thus, limiting the maximum number of crosslinks which can form per molecule.

Both the difunctional and trifunctional crosslinks occur at these sites. The quarter staggered, head-to-tail configuration of molecules within fibrils allows for alignment of crosslinking residues on adjacent collagen molecules and divalent crosslink formation. While the formation of difunctional crosslinks is limited to collagen molecules in head-to-tail, quarter staggered configuration, a trivalent crosslink permits head-to-head or zero staggered components in or between fibrils [8,26].

The first step in crosslink formation is the conversion (by lysyl oxidase} of the epsilon amino group of selected lysine or hydroxylysine residues to the corresponding aldehyde {allysine or hydroxyallysine). Crosslinks then form by spontaneous reaction of an allysine or hydroxyallysine with an unmodified lysine or hydroxylysine residue on an adjacent polypeptide chain. Two pathways of crosslink formation for fibrillar collagens have been described, one arising from crosslinks formed with allysine and the oilier from hydro xyallysine [8]. The initial crosslinks formed on either pathway are difunctional and are usually described as reducible crosslinks because they possess Schiff base double bonds which can be rcductively labeled [7, 8,26]. Reducible crosslinks vary in their stability, occur transiently and can be considered intermediate products. With maturation divalent crosslinks disappear from many tissues and may be replaced by mature, non-reducible crosslinks. The crosslink pathway which apparently predominates in mammalian heart collagen is the one based on hydroxyallysine [10,21]. The mature crosslinking residues on Ihe hydroxyallysine pathway are trivaient, 3-hydroxypyridinium (HP) residues and lysyl pyridinium, with the latter present in negligible amounts in most tissues except bone. HP is putatively formed in a precursor-product manner from the condensation of two reducible ketoamine crosslinks, a mechanism of formation that is confirmed by the stoichiometric relationship between Ihe disappearance of ketoimine crosslinks and the accumulation of HP in tissues [16]. The progression of crosslinks from divalent to trivaient forms during maturation is significant because multivalent crosslinks have the potential to markedly increase the strength of the myocardial interstitium by linking together adjacent fibrils as well as individual collagen molecules [8, 26].

The progressive nature of collagen crosslink biosynthesis does not necessarily mean that there is a steady, irreversible progression of lysine aldehyde-derived crosslinks from less to more mature forms in the heart. While there is good correlation between maturation of heart collagen crosslinks and chronological age, it is also apparent thai rate of crosslink formation and directional shifts in the concentration of mature crosslinks, irrespective of age, can be altered [20, 32, 33, 34, 39].

Possible Mechanism Regulating Crosslink Formation

Relatively little is known of the mechanisms which regulate crosslinking in muscle, including heart [26]. Lysyl oxidasc, (he only known enzyme involved in the crosslinking process, requires copper as a cofactor. In studies with severely copper deficient swine [34] and rats [9] depressed non-reducible crosslinking in the myocardium was observed. However, in the absence of severe copper deprivation or inactivation of lysyl oxidase by lathyrogens, it is unlikely that moderate perturbations in lysyl oxidase activity affect crosslinking. In tissues where it has been possible to measure lysyl oxidase activity, such as skin [29], the concentration of lysyl oxidase far exceeds minimal requirements for crosslink formation. Thus, even large variations in lysyl oxidase activity may not affect crosslinking patterns in the heart, a tissue in which activity of this enzyme has not successfully been determined [34J.

Levels of lysine hydroxylation influence crosslinking patterns in tissues, including proportions of HP to its ketoamine precursor [12] and the ratio of lysine aldehyde lo hydroxylysine aldehyde crosslinks [17]. There is variability in levels of lysine hydroxylation among collagen types and among different tissues; however, levels or degree of variability in myocardial collagen lysine hydroxylation have not been reported.

The observation that crosslinking residues on adjacent molecules or fibrils must be precisely aligned for crosslinking to proceed, suggests that spatial relationships between types I and III collagen may be a controlling factor in crosslink formation [26, 35], As noted above, populations of collagen molecules and fibrils may exist with varying frequencies of head-to-head (zero stagger) and head-to-tail (quarter stagger) components. Due to the restriction of all known crosslinks to four sites, molecule and fibril orientation would be expected to affect both divalent and trivalent crosslink formation [11].

A mechanism by which spatial relationships among collagen molecules may be regulated involves the binding of decorin to fibrillar collagen [35, 41]. Decorin is a small chondroitin su I fate/derma tan sulfate proteoglycan that associates with collagen types I and III [24,31]. Interactions between the core protein of decorin and collagen govern Ihc rate and extent of collagen fibrillogcncsis [36]. The binding of decorin to collagen is periodic and axial along the fibril, with decorin protein binding sites corresponding to the lateral shift or stagger of one collagen molecule to another [24]. Studies using dee or in-deficient mice have demonstrated thai this proteoglycan is a key regulator of collagen fibrillogenesis [6]. Alterations in decorin expression and accumulation do influence the pattern of fibril formation and, thus, would be expected to influence crosslink formation [35]. In both the avian Low Score Normal model of skeletal muscle weakness [35] and following surgically induced myocardial infarction in the rat [40], the rapid accumulation of non-reducible crosslinks is preceded by an increase in tissue decorin levels. We hypothesize that decorin/collagen interactions resulting in alterations in orientation of molecules and fibrils and the alignment ofcrosslink sites is a primary mechanism regulating crosslink formation [35].

Myocardial Collagen Crosslinking with Development and Aging

The only longitudinal studies, to our knowledge, which have evaluated changes in myocardial collagen crosslinking associated either with early development, or from young adulthood into senescence, have done so with pig and rat models, respectively [21, 32]. The findings from these studies support the concept that crosslinking in the heart is affected by aging, and, specifically, by age-associated changes in the rates of collagen synthesis, degradation and turnover. In pigs sacrificed at one day of age and at intervals up to 32 weeks of age, collagen crosslinking (HP concentration) in combined left ventricular free wall and septum increased from 0.3 moles HP/mole collagen at birth up to 1.23 moles/mole collagen in the young mature animal. As can be seen in Figure 1, HP values remained fairly constant during the first 8 weeks of life but, in the subsequent two month period crosslinking rose rapidly, and then leveled off somewhat over the final two month period of the study. Simultaneous measurement of collagen concentration in the hearts of these same pigs revealed that, while collagen concentration at birth is already 70

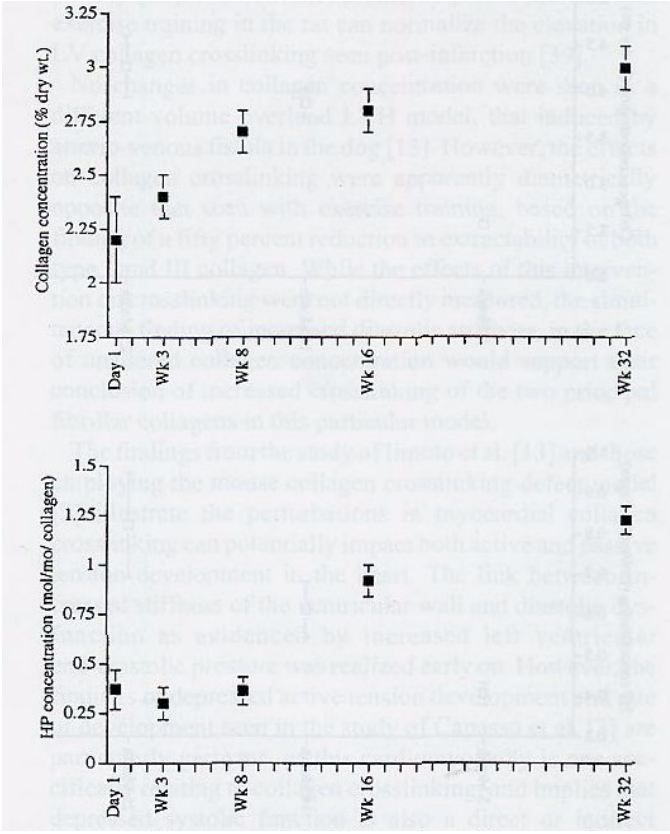

Figure 1. Developmental changes in collagen and hydroxypyridinoline (HP) crosslink concentration in swine left ventricular myocardium from day 1 to 32 weeks of age.

percent of that seen in young adulthood, HP concentration is only 30 percent of young mature values. Interestingly, the greatest increase in collagen concentration occurred between 3 and 8 weeks of age, the time period immediately prior to the documented rapid increase in crosslinking. It would also be of interest to correlate the rapid changes in crosslinking of collagen seen in the hearts oflhcsc immature pigs with developmental changes in systemic blood pressure during (his same period.

The results from studies assessing changes in left ventricular collagen crosslinking from maturity to senescence using the rodent model [32, 33] indicate that crosslinking of fibrillar collagens in the heart continues to increase throughout life, at least in Ihe rat (Figure 2). This increase, which is fairly linear, occurs both from five (young adult) to fourteen months of age (middle-age), as well as from fourteen to twenty-five months (senescence). This increase also occurs despite a gradual decline in mRNAs for both types I and III collageninLVfrecwallbi.it not septum over this same twenty month period [32], These latter findings indicate that intracellular synthesis rate of collagen does not necessarily predict extent of crosslinking in the ECM as collagen turnover has been shown to decline slowly with aging in heart tissue [18].

The findings from these two longitudinal studies, combined with single values obtained in other studies using

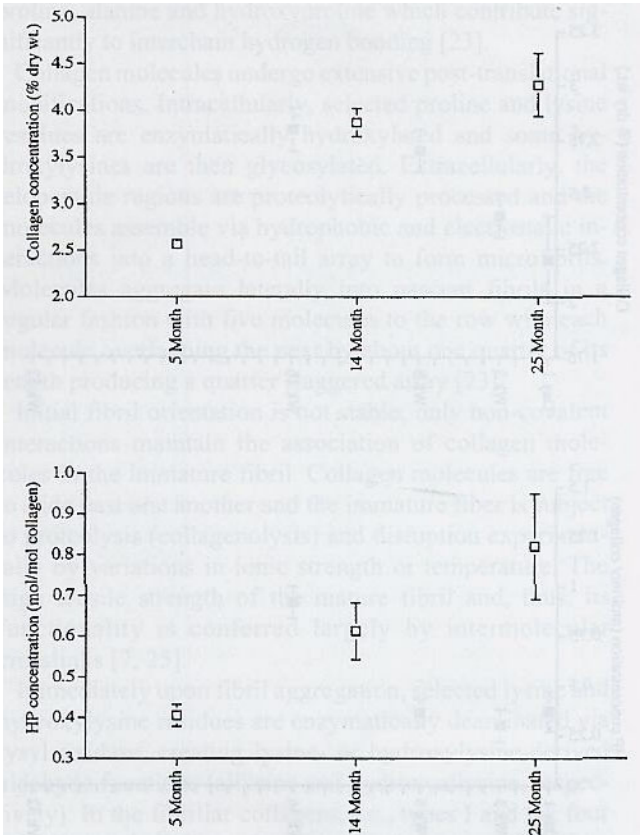

Figure 2. Collagen and hydroxypyrinoline (HP) crosslink concentration in left ventricular myocardium from young adulf (5 month old), middle-aged (14 month old), and senescent (25 month old) rats.

different species, also point out the fad that there are species-related differences in myocardial crosslink concentration. The hearts from larger mammals are more heavily crosslinked than those from smaller mammals. Maximal HP concentration values in the senescent rat myocardium are 0.7-0.8 moles/mol collagen. In young mature swine and adult humans normal concentrations are 1.23 and2.0 moles HP/molcollagen,respectively [10,2i], Greater crosslink concentrations in the left ventricle of larger mammals may reflect the greater requirement for force development in the face of higher peripheral resistance to forward output. The differences in left ventricular crosslink concentration may likewise play a role in determining intrinsic heart rate in different sized species. Thus, the rapid resting heart rate in Ihe rat (400 beats/mill) could conceivably be impossible with the degree of crosslinking measured, for example, in the human.

Functional Consequences of Altered Collagen Crosslinking in Left Ventricular Hypertrophy (LVH)

One of the difficulties in evaluating the significance of I changes in crosslinking of collagen coincident with LVH j and/or altered LV function is the overall paucity of data. The scarcity of data related to this EMC parameter rein-1 forces Eyre's observation that crosslinks are not abundant relative to the bulk of amino acids (< 0.1 to 1 residue per 1000 amino acid residues) and are, consequently, difficult I to measure [7]. Where changes in collagen crosslink concentrations have been either measured [10, 20, 21, 32,33,1 34], or assumed [4, 13, 30], as in the sex-linked defecl al the mottled loeus in mice, simultaneous measurements of j pump function and LV contractility together with collagen phenotype expression, amounts and type I / type III ratios have not been made. In other studies where a considerable I amount of data showing perturbations of the ECM in I various pathological hypertrophy models of the heart are I presented it is difficult or impossible to separate out the! respective contribution of the aforementioned ECM pa-| rameters to overall LV dysfunction. A further eomplica-l tion is the fact that LV dysfunction in many cases is I associated with both intracellular as well as extracellular I myocardia! perturbations. In these situations it is some-1 times unclear which is the primary and which is the secon-1 dary lesion. Finally, in some pathological models [20], but I not others [34], both amount of collagen and the degree loj which it is non-reducibly crosslinked increase, again mak-l ing it difficult to separate the relative contribution of these | different changes on LV function.

Studies which have evaluated ECM changes associated! with various LVH models have looked at volume- [13] and] pressure-overload including hypertension [5], idiopathiq cardiomyopathy [10], copper deficiency [9, 34], adraiaiJ stralion of selected hormones such as thyroxin [38] and] norepinephrine, myocardial infarction [20] and exercisi training [33]. However, in the bulk of these studies evalu-1 ation of the ECM has been limited to measurement off changes (or lack thereof) of collagen concentration assoj

cialed with the overall hypertrophy process. In some of these same studies expression of message for Ihe two principal fibrillar collagens has also been evaluated. Where (he only interstitial parameter being evaluated is concentration of collagen, and where this has been seen to increase, investigators have concluded that this change alone is responsible for the elevated stiffness of the ventricular wall [5], either assumed or sometimes measured, which ultimately leads to heart dysfunction and/or failure. The only cardiomegaly models which, to our knowledge, have simultaneously measured collagen concentration and ihe extent to which it is crosslinked are those examining copper deficiency, cardiomyopathy, myocardial infarction and exercise (raining [10, 20, 32. 34]. In the first of these models, that induce by copper deficiency, the putative inhibition of the cu pro-dependent enzyme lysyl oxidase, the controlling enzyme for collagen crosslinking, mimics the crosslinking defect mouse model [9, 30]. Both of these models have been associated with aortic and/or ventricular aneurysms [1*4]* emphasizing the role that the crosslinking *perse* plays in normal vascular and ventricular function. Interestingly in the porcine copper-deficient model of ventricular hypertrophy [34], the reduction of collagen crosslinks and putative increase in wall stress, which then provides the stimulus for individual myocyte hypertrophy, occurs without an increase in collagen concentration, al-Ihough the relative fraction of type III collagen increased some 25 percent above that seen in control hearts. Whether collagen synthetic and degradative rates were also affected by inhibiting HP crosslinking was not evaluated. Although ventricular function measurements were also lacking in these pigs, compromised myocardial mechanics and function were documented in the hearts of rats employing the same model [25]. increases in chamber size and associated wall stress are I ikewise thought to be a major stimulus for the dramatic alterations in ECM in remaining viable myocardium seen following myocardial infarction [20], although this insult is known to trigger other hormonal stimuli which also target the flbroblast cell. In this pathological LVH model, unlike the copper-deficient model, a rapid interstitial fibrosis is seen in both the LV free wall and sepnim with regional differences perhaps being related to differences in wall stress changes between the two regions. In the rat infarct model, collagen concentration more than doubled in LV frecwall by 13 wk post-Mi implying a rapid increase in collagen synthetic rate and/or inhibition of collagenase activity (not measured). Even more impressive, perhaps, is the fact that this newly synthesized collagen was also very heavily crosslinked, and total HP crosslinks in LV freewall, based on both the total amount of collagen and number of crosslinks expressed as mol HP/mol collagen increased some 3.6-fold. Whether or not extent of change in either or both of these ECM parameters plays a role in determining eventual outcome with respect to LV function is, as yet, unknown. However, an interesting comparison is the spontaneously hypertensive rat (SHR) model, a pressure-overload model, where the extent of perturbation of the ECM correlated directly with LV function [5]. In this model the progression from compensated LVH to failure was associated with marked myocardial fibrosis although, unfortunately, measurements of collagen phenotype or crosslinking were not made.

A contrasting physiological LVH model is the chronic volume-overload model produced by exercise-training. Again using the rat as the model, a small (10-15%) but significant cardiac hypertrophy is produced irrespective of age of the animal [33], Interestingly, this intervention affects neither ventricular collagen amount, nor ihe degree to which it is non-reducibly crosslinkcd in the young mature adult aniinal. However, the same intervention in senescent rats, which by 23 months of age have increases in both LV collagen concentration and the extent to which it is crosslinked, causes a significant reduction in the HP crosslink without affecting overall collagen concentration. Whole body \O2max and, by implication maximal cardiac output, were also improved in these trained senescent rats compared to their sedentary peers [40]. Taken together these findings indicate that exercise-induced reductions in the elevation of HP crosslinking in the heart associated with aging, may attenuate the increased stiffness also seen with this process, allowing for improved ventricular function of the senescent trained hearts as documented previously [15], Furthermore, preliminary data indicate that exercise training in the rat can normalize the elevation in LV collagen crosslinking seen post-infarction [39],

No changes in collagen concentration were seen in a different volume overload LVH model, that induced by arterio-venous fistula in the dog [13], However, the effects on collagen crosslinking were apparently diametrically opposite that seen with exercise training, based on the finding of a fifty percent reduction in extractabilily of both type I and III collagen. While the effects of this intervention on crosslinking were not directly measured, the simultaneous finding of increased diastolic stiffness, in the face of unaltered collagen concentration would support their conclusion of increased crosslinking of the two principal fibrillar collagens in this particular model.

The findings from the study of limotoetal. [13] and those employing the mouse collagen crosslinking defect model [3] illustrate the perturbations in myocardial collagen crosslinking can potentially impact both active and passive tension development in the heart. The link between increased stiffness of the ventricular wall and diastolic dysfunction as evidenced by increased left ventricular end-diastolic pressure was realized early on. However, the findings of depressed active tension development and rate of development seen in the study of Capasso et al. [3] are particularly germane, as this cardiomyopathy is one specifically relating to collagen erosslinking, and implies that depressed systolic function is also a direct or indirect consequence of this particular perturbation of the ECM.

To our knowledge the recent study examining explant heart tissue from patients with idiopathic dilated

cardiomyopathy is the only human study which has specifically evaluated collagen cross! in king in the etiology of heart failure in humans [101, '" those patients collagen concentration doubled whereas HP concentration was only fifty percent of that seen in autopsy specimens from individuals that had died from causes unrelated to heart disease. Again the findings point to a defect in collagen metabolism associated with this particular pathological LVH thai may be specific to mature crosslinking of newly synthesized collagen in the ECM. While the authors do not speculate as to the cause oflhc apparent paradox of fibrosis without increased stiffness or stability, the findings could bc'explained by a defect in the crosslinking pathway(s) which leads to the dramatic increases in collagen concentration and collagenase activily with this pathology.

Collagen crosslinking or HP concentration is species-dependent, being lower in the hearts of small vs. large mammals which makes it inversely related to resting heart rate across species. In the rat, the only species to our knowledge in which there are data jn the literature across the life-span, the HP crosslink approximately doubles from 5 to approximately 24 months of age, a change which is similar to thai seen in collagen content in the heart as estimated by hydroxyproline concentration. Importantly, both increases and decreases in crosslink concentration may be seen in different pathologies of the heart, and, with ihe exception of the physiologically-induced hypertrophy of exercise, appear lo have deleterious consequences in terms of ventricular function. Thus, defects in crosslinking are implicated in the subsequent hypertrophy of ihe ECM (fibrosis) and may also be involved in stimulating abnormal growth of individual myocytes. Whether it is the documented increase in crosslinking that ultimately leads to myosin isoform shifts and chronotropie insufficiency in the rat myocardial-infarclion LVH model is an additional point for conjecture.

In conclusion, significant advances in our understanding of collagen crosslink chemistry have been made. However, mechanisms regulating rate of crosslink formation and accumulation in tissues, including heart, remain elusive. Treatment of a variety of cardiac pathologies involving the ECM will require a deeper understanding ofthe dynamics of collagen metabolism, particularly with respect to crosslinking.

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